



Extensive dry heating-induced changes in physicochemical and immunological properties of whey proteins

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in physicochemical and immunological
properties of whey proteins**

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

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Thesis

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof. Dr APJ Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Tuesday 20 December 2016

at 1.30 p.m. in the Aula.

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Extensive dry heating-induced changes in physicochemical and immunological properties of whey proteins

146 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)

With references, with summary in English

ISBN: 978-94-6343-001-2

DOI: 10.18174/394749

List of abbreviations

AGEs, advanced glycation end products
ALA, α -lactalbumin
ANS, 8-anilino-1-naphthalenesulfonic acid
 a_w , water activity
BLG, beta-lactoglobulin
BMDCs, bone marrow-derived dendritic cells
BSA, bovine serum albumin
CD, circular dichroism; BLG, beta-lactoglobulin
CMA, cow's milk allergy
CML, carboxymethyllysine
DC, dendritic cells
DH, degree of enzymatic hydrolysis
DTNB, 5,5'-dithiobis (2-nitrobenzoate)
ELISA, enzyme-linked immunosorbent assay
EPIT, epicutaneous immunotherapy
FITC, Fluorescein isothiocyanate
Foxp3, forkhead box protein 3
GI, gastrointestinal
IgE, immunoglobulin E
IL, interleukin
LAC, lactose
LMW, low molecular weight
LPS, lipopolysaccharide
MFI, mean fluorescence intensity
MLNs, mesenteric lymph nodes
MRPs, Maillard reaction products
OIT, immunotherapy
OIT, oral immunotherapy
OPA, o-Phtalaldehyde
OPD, o-phenylenediamine
OVA, ovalbumin
PBST, PBS-Tween
qPCR, Quantitative real time PCR
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC, size exclusion chromatography
SGF, simulated gastric fluid
SIF, simulated intestinal fluid
SIT, allergen-specific immunotherapy
SLIT, sublingual immunotherapy
sRAGE, soluble advanced glycation end products receptor
TBS, Tris-buffered saline
tDCs, tolerogenic DC
TMB, 3,3',5,5'-tetramethylbenzidine
TNF- α , tumour necrosis factor- α
Treg cells, regulatory T cells
TX, Triton
WPC, whey protein concentrate
WPI, whey protein isolate

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Chapter 1

General introduction



Food allergy

Food allergy is an adverse and reproducible immune-mediated reaction on exposure to a given food. It differs from food intolerance, such as lactose intolerance, which is an adverse reaction usually with allergy-like symptoms, but without proven involvement of the immune system. Based on the immunological mechanism involved, food allergy can be further classified in immunoglobulin E (IgE)-mediated food allergy and non-IgE-mediated food allergy. The IgE-mediated food allergy, also called type 1 food allergy, occurs within minutes to hours after the exposure to the corresponding allergen. Non-IgE-mediated food allergy is mediated by other types of immunoglobulins, i.e. IgG, IgA, and occurs hours or days after exposure to the allergen [1]. Food allergy as referred to in this thesis represents IgE-mediated food allergy.

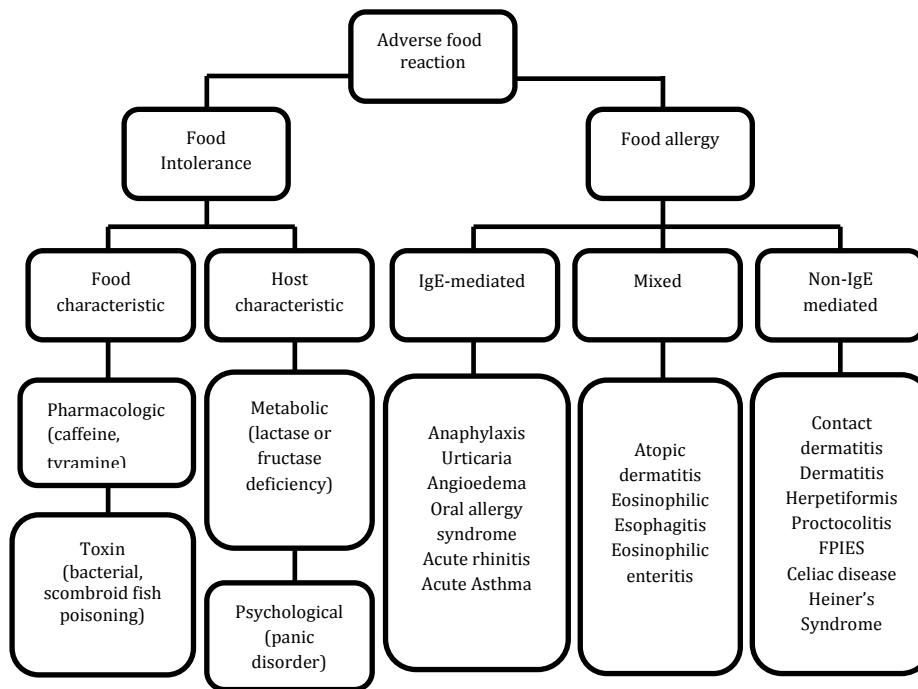


Figure 1.1 Classification of adverse reactions to foods (adapted from Cianferoni et al. [2]).

The prevalence of food allergy varies with geography, local eating habits, and age of the testing population. Food allergy affects more than 1-2% but less than 10% of the population [3]. Several health surveys indicated that this prevalence has increased in the past decades [4, 5]. Infants less than 3 years of age are among the most susceptible population. In infancy, the gut barrier is not yet fully developed. The secretion of proteolytic enzymes and gastric acid are lower compared to adults, increasing the amount of “immunologically” intact allergens that are capable to elicit immune responses. The prevalence decreases over the first decade of life. About 75% of the infants who have IgE-mediated cow’s milk allergy (CMA) in their first year of life naturally outgrow their allergies by the second decade, whereas peanut, nuts, and seafood allergies are thought to be never outgrown [6]. Cutaneous, respiratory, and gastrointestinal symptoms expressed by allergic patients are well-documented (Fig. 1.1). In addition, patients might have cardiovascular symptoms, including hypotension, vascular collapse, and cardiac dysrhythmias [7]. Thus, suffering from food allergies greatly affects the quality of life of allergic subjects.

CMA

Cow’s milk contains numerous nutrients, such as protein, calcium, phosphorus, vitamin B12 and vitamin D. As a main substitute of human milk, cow’s milk proteins represent the first source of antigens encountered in large quantities in infancy. CMA is the leading cause of food allergy, especially in infants and children. The incidence of CMA was reported to be 1.9 to 2.8% of infants under the age of 2 in northern Europe, but it was reported to decrease to approximately 0.3% in children older than 3 [8]. In the USA, the reported prevalence of CMA is 3.8% in infants up to 12 months of age [9]. Life-threatening allergic reactions to cow’s milk allergens may also occur, but are fortunately rare [10]. In most of the regions, information on the prevalence of CMA is not available, and there are no national or regional guidelines for CMA, but it is well acknowledged that CMA is a global challenge and there is a shortage of international systematic guidelines regarding the management of CMA.

Cow's milk allergens

Cow's milk contains about 30-35 g of proteins per litre. More than 200 proteins were identified in bovine milk [11]. Acidification of bovine milk to pH 4.6 results in two protein fractions: whey, representing about 20% of the proteins, and casein, representing about 80% of the proteins. Among the whey proteins, β -lactoglobulin (BLG) is the most abundant, and is also involved in most allergic responses induced by cow's milk. Most cow's milk allergens could be ingested and hydrolysed to amino acids or peptides by proteases, however, 2% of the protein allergens ingested by adults remain intact and are capable to induce immune responses [12].

About 82% of the CMA patients are sensitive to BLG [13]. BLG is a globular protein with a molecular weight of 18.3 kDa and exists mainly in the form of a 36-kDa dimer at neutral pH. It has two disulphide bridges and one free thiol group, which is hidden within the folded protein. Several properties of BLG, such as having no homologous counterpart in human milk, its relatively high resistance to acid and proteolytic hydrolysis, its membership of the lipocalin family that shares a similar tertiary structure and a high affinity for hydrophobic ligands, render it potentially highly allergenic. Epitopes, the conformational structures or linear amino acid sequences on allergens that can interact with an antibody, on BLG have been detected in patients with persistent allergy. BLG IgE epitopes were reported to be located in the sequence at amino acids 1-16, 31-60, 67-86, and 127-156 [14]. Fragments 41-60, 102-124, and 149-162 were reported to be epitopes in another study [15]. Discrepancy in the epitopes among different studies may result from the differences in the sera used for epitope mapping. Some dominating and well-documented epitopes could be obtained from the overlapping sequences as reported in different studies. Information on these epitopes offers a number of new possibilities for the diagnosis and treatment of CMA. For instance, allergen-specific immunotherapy (SIT) by using T-cell epitopes, which has a reduced capacity (vs whole allergen) to cross-link IgE on effector cells, represents a more safe and efficient approach than using native protein.

Management of CMA

The current common management of food allergy is mainly limited to strict allergen avoidance, nutritional counselling, and managing severe allergic reactions with self-

injectable epinephrine. Management of CMA is of special importance to the allergic subjects. Cow's milk is the main substitute of human breast milk for infants and young children. A cow's milk substitute, such as the partially hydrolysed cow's milk-based formula, extensively hydrolysed cow's milk formula, amino acid-based formula, and soy-based formula, should be recommended to the infants who suffer from CMA. Such substitutes have to fulfil the general nutritional requirements to support growth and development of infants until the introduction of complementary foods. Among these substitutes, the hydrolysed formula is the first choice as an alternative to cow's milk.

Both food allergen-specific and nonspecific approaches have been pursued to treat food allergies. Nonspecific approaches include monoclonal anti-IgE antibodies, which might increase the threshold dose for food allergens in subjects with food allergy, and a Chinese herbal formulation were investigated in clinical trials [13]. Several allergen-specific approaches, including oral immunotherapy (OIT), sublingual immunotherapy (SLIT), and epicutaneous immunotherapy (EPIT) with food allergens, have been pursued to treat food allergy [16].

As an alternative approach of allergen-specific treatment, addition of extensively heated (baked) milk and egg to patients' diets seems to represent an alternative oral immunotherapy and is already changing the paradigm of strict allergen avoidance for patients with food allergy in the USA [17, 18]. Kim et al. reported that approximately 75% of children with CMA can tolerate baked milk products, e.g. as milk-protein containing muffin and baked cheese. In addition, subjects who incorporated baked milk into their diets were 16 times more likely than the comparison group to obtain tolerance to unheated milk over a median of 37 months ($p < 0.001$) [17]. Apart from the study of Kim et al., there is emerging evidence (Table 1.1) that egg or cow's milk allergic subjects who can tolerate baked allergens are more likely to develop tolerance than the baked allergen reactive children. As shown in Table 1.1, the baked allergens were prepared in similar temperatures in the clinical studies. The pH and water activity (a_w) of the system were not reported. Results showed that the proportion of subjects tolerating baked milk depends on the age and the methods used to prepare baked samples.

In the past decade, these studies suggested a promising use of baked allergens in the management of CMA [19]. Baked milk mentioned in these studies represents the milk

proteins contained in baked foods, such as muffin, cookie, and baked cheese. Compared to heating in solution, the baked proteins underwent extensive heat treatment in a relatively dry, high temperature, and low a_w condition. The baking process starts from a homogeneous dough with higher a_w , undergoes nonuniform water loss and a varying temperature distribution, and ends up with a product with extensively heated surface and a moderately heated inner part.

Based on the results of these observational studies, it can be expected that the baking process modifies the properties of milk proteins in a way differing from heating in wet conditions (i.e., proteins in aqueous solutions), and may confer immunomodulatory properties on milk proteins in baked foods. However, some research questions need to be addressed before such baked foods can be widely used for clinical practice. First, several studies on immune tolerance to CMA and egg allergy induced by baked proteins have been questioned because of the absence of proper control groups, making it is uncertain whether the observation of induction of immune tolerance is a result of baked allergen consumption or a consequence of the natural course of egg and milk allergies [20]. Second, the effects of such an extensive heating in dry condition on the physicochemical properties of cow's milk allergens remain unclear. Third, the functional compounds in dry heated samples, and immunomodulatory cells or cytokines, which may play a role in the development of baked allergen-induced immune tolerance, are still unknown. This thesis focuses on the second and third questions, aimed at determining the influence of extensive heating of milk proteins in relatively dry conditions on their chemical-physical and immunological properties.

Extensively heated protein and CMA

Impact of extensive heating on the physicochemical properties of cow's milk allergens

Heat treatment is the most used process in the food industry. The effect of heat treatment on cow's milk proteins was extensively studied in solution under controlled physicochemical conditions [24, 25]. In contrast, a limited number of studies focused on the dry heating of cow's milk proteins, and it was reported that the modifications in the properties of milk proteins during dry heating cannot be extrapolated from results obtained in solution [26].

Table 1.1 Baked milk clinical studies (adapted from Leonard et al. [19]).

Type of study	Inclusion criteria	Age of population	Baking method	Rate of tolerance % (fraction of subjects)
Kim et al. [17] Prospective	Allergic reaction to milk in past 6 months and positive testing (SPT or sIgE), or highly predictive testing (sIgE >5 kUA/L if <2 years or >15 kUA/L if >2 years, or SPT wheal 8 mm)	Median 6.6 years	1.3 g of milk protein from nonfat dry milk powder (70/88) after a median 177 °C for 30 min.	80% (68/91); 80% (70/88) after a median follow-up of 37 months
Bartnikas et al. [23] Retrospective	Allergist-documented history of allergic reaction to milk and/or positive testing (SPT or sIgE)	Median 8.1 year	1.3 g of milk protein from nonfat dry milk powder in a muffin baked at 177 °C for 30 min.	83% (29/35)
Caubet et al. [22] Prospective	Allergic reaction to milk in past 6 months and positive testing (SPT or sIgE), or highly predictive testing (sIgE >5 kUA/L if <2 years or >15 kUA/L if >2 years, or SPT wheal 8 mm)	Baked milk reactive: 8 years; Baked milk tolerant: 7.5 years	1.3 g of milk protein from nonfat dry milk powder in a muffin baked at 177 °C for 30 min.	69% (83/121)
Meihr et al. [21] Prospective	Allergic reaction to milk in past 12 months and positive testing (SPT or sIgE), or SPT wheal >7 mm if >2 years or >5 mm if <2 years	Median 5.3 years	0.5 g of milk protein in a muffin baked at 180 °C for 20 min	73% (51/70)

SPT skin prick test, sIgE specific IgE

Upon heating in the absence of reducing sugars, the native BLG dimers progressively dissociate into native monomers. Aggregates can then be formed via intermolecular thiol-catalysed disulphide bond interchange, non-covalent interactions and thiol-thiol oxidation [25]. It was reported that the native monomeric proteins were also capable of converting to non-native monomers via intramolecular disulphide bond interchange [27]. Covalently bound aggregates were formed from these non-native monomers. Heat treatment-induced conformational modifications of α -lactalbumin (ALA) were mainly pH and calcium dependent. In the absence of calcium ions, extensive heating gave rise to non-native monomers, dimers, trimers, and larger aggregates [28]. When mixtures of BLG and ALA were heated, BLG aggregates were formed via disulphide bonds, while ALA aggregates were formed via both disulphide bonds and hydrophobic interactions. The heterogeneous aggregates of ALA and BLG were formed mainly by disulphide bonds and to a lesser extent by non-covalent bonding. In the early stages of heating, aggregates contained more BLG, whereas in the later stages, they contained equal amounts of both proteins [29].

Upon heating in the presence of reducing sugars, the Maillard reaction occurs between the ϵ -amino group of lysine residues in a protein and the carbonyl group in a reducing sugar. The Maillard reaction is one of the most frequent chemical protein modifications during industrial processing which leads to changes in flavour, colour, texture, and nutritional value. It is a complex reaction that can be subdivided in three stages: the early stage, the advanced stage, and the final stage. In the early stage, the so-called Amadori product (lactulosyllysine in milk) is formed via the formation of a Schiff's base and the Amadori rearrangement. The early stage of the Maillard reaction can be detected by measuring furosine, which is a relatively stable compound formed from the Amadori compound upon acid hydrolysis. In the advanced stage, the Amadori products are broken down to numerous fission products of the sugar-amino compound [30]. Advanced glycation end products (AGEs) are generated from Amadori products, and play important roles in several pathophysiological and immune responses. AGEs constitute a group of heterogeneous compounds (e.g. pentosidine, carboxymethyllysine (CML), imidazolone, pyrrolin, GA-pyridine, etc.) [31]. The generation of AGEs is affected by heating conditions, such as nutrient composition, temperature, heating time, amount of moisture, and pH of the food matrix [32]. AGEs may interact with its receptors and elicit immune responses via a NF- κ B mediated

pathway, but study on its immunological properties is insufficient [33]. The final stage of the Maillard reaction consists of the condensation of amino compounds and sugar fragments into brown pigments containing polymerized protein, the structure of which is largely unknown. The Maillard reaction affects protein allergenicity in several ways, including destruction of conformational epitopes, and chemical reactions with the food matrix that could lead to limited availability of the protein to the immune system or proteolytic hydrolysis. Contrarily, new epitopes may be formed by the Maillard reaction, and the changes in the resistance of modified allergens to enzymatic digestion may influence linear epitope and subsequently affect the allergic responses.

A_w , pH, and heating duration are the main factors affecting the outcomes of extensive dry heating. Dry heating is usually performed at pH 7-9, but some results indicated that heat treatment at acidic pH can result in modifications in the protein functionalities [34]. In a study of Gulzar et al., whey protein isolate (WPI) was dry heated at a_w 0.23 and various pH values (2.5, 4.5, and 6.5). The heat treatment led to a decreased solubility, and formation of aggregates with increased size, with the increase of pH. Intermolecular disulphide bonds were preferably formed at pH 2.5, while disulphide bonds and other covalent cross-links were formed at higher pH values [35]. Compared to heating in solution, the kinetics of lactosylation of milk proteins would be greatly accelerated in such dry heating conditions [36]. In addition, it is notable that dry heating at a relatively low temperature (50°C) does not significantly alter the native structure of the BLG, whereas glycation in solution induces modifications in the structures leading to dissociation of the dimer and formation of compounds with higher molecular weight [37]. In regards to extensive dry heating, further studies need to be performed to evaluate if the native-like aggregates can also be generated.

Effects of heating on the allergenicity of milk allergens

The heat treatment-induced decrease in the solubility of allergens may reduce the accessibility of allergens to the immune system. In the case of peanut protein, the resulting insoluble material appeared as a protein smear in electrophoresis gels, this insoluble material was reported to possess enhanced IgE-binding capacity [38]. There is no report on the allergenicity or IgE-binding capacity of thermally-induced insoluble milk proteins. Heat treatment may induce the formation of soluble

aggregates of milk proteins. Increasing evidence shows that aggregation could lead to modifications in the proteolytic digestibility of milk proteins [39, 40]. Consequently, milk protein aggregates and their digestion products may possess modified allergenic properties compared to non-glycated proteins, and thus play important roles in the sensitization stage of cow's milk allergy [41]. In addition, the aggregation of allergens redirects uptake to Peyer's patches, significantly promoting the production of Th2-associated antibodies and cytokines in mice compared to their native counterparts [41].

Several competing mechanisms determine the final allergenicity of glycated proteins. Interactions with sugars may decrease the accessibility of epitopes, whereas the thermal-induced unfolding of protein allergens or the breakdown of the Maillard reaction products may expose the conformational epitopes within the protein molecules. In addition, glycation can potentially induce the formation of new epitopes (neoepitope) and thereby increase the IgE-binding capacity of allergens [42]. Contradictory results have been reported so far about the effects of glycation on the allergenicity of glycated proteins [43, 44]. In some cases, glycation of proteins enhances their IgE-binding capacity [44, 45], whereas in some other studies, glycation was reported to reduce the IgE-binding capacity [46, 47], or to have no influence [46, 48]. Compared to the protein heated without reducing carbohydrates, glycated protein may possess different properties conferred by carbohydrate moieties. It is notable that carbohydrates moieties in glycosylated allergens have also been shown to affect their affinity for specific IgE [49], but whether the carbohydrate moieties in glycated proteins participate in allergic reactions and in suppressing the immune responses remains unknown.

The allergenicity of AGEs has been rarely studied. Chung et al. reported that AGEs could be in part responsible for the increased IgE-binding capacity of roasted peanut allergens, but whether the AGE adducts could induce an allergic reaction remains elusive [45]. It was reported that BLG heated with lactose acquired a 100-fold increase in skin reactivity [50]. No general conclusion can be drawn since a limited number of studies focused on the allergenicity of AGEs. However, these results already indicate the importance of AGEs and/or other Maillard reaction products in food allergy.

The possible mechanism of oral tolerance induced by extensively heated allergens

Many mechanisms have been suggested to explain the development of oral tolerance, including clonal energy, clonal deletion, and active regulatory processes (Fig. 1.2). The most popular and important approach of the development of immune tolerance is the active regulatory processes (pathway c in Fig. 1.2), which is associated with the generation of inducible or adaptive Treg cells [51]. Increasing evidence links oral tolerance to the function of mucosal DCs, which are professional APCs, to induce forkhead box protein 3 (Foxp3)-positive Treg cells in mesenteric lymph nodes (MLNs) [52]. TR1 cells, a population of CD4⁺ T cells that produce IL-10, are formed in the presence of the immunomodulatory cytokine IL-10 and proceed themselves to produce IL-10, thereby contributing to the induction of Treg cells and tolerogenic DCs. Apart from professional APCs, the nonprofessional APCs, such as intestinal macrophages, also have the ability to induce Foxp3⁺ Treg cells in an IL-10-, RA-, and TGF- β -dependent setting [53]. These studies indicated the important role of DCs in the development of immune tolerance. In addition, development of immune tolerance is dose-dependent. Repeated challenges with low dose of allergens are thought to contribute to the development of Treg cells, which depress the immune tolerance through secreted or cell-bound regulatory cytokines, such as IL-10 and TGF- β [54]. High dose challenge induced tolerance is mediated by lymphocyte anergy or clonal deletion [55]. Heat treatment changes the accessibility of allergens to the immune system by altering their solubility and digestibility. The heated allergen-induced tolerance could therefore be, at least partly, explained similarly as the dose-dependent mechanism.

Heat treatment induces aggregation and glycation in the mixtures of protein allergens and carbohydrates, leading to changes in the particle size of the allergens. Aggregates and monomers of allergens may then get access to the intestinal immune system via different size-dependent pathways [56]. Therefore, it can be speculated that extensively heated allergen induced tolerance would be already initialized in the uptake process of allergens and the subsequent interactions with MLNs, which is considered as the major site of antigen loading and T-cell recognition [57]. Furthermore, tolerogenic dendritic cells with regulatory activities have been

identified in the MLNs [58]. These cells induce the development of Tregs during aggregated allergens are selectively uptake by M cells in Peyer's patches and transferred to neighbouring APCs. Even so, it is well-acknowledged that the Peyer's patches and conventional M cells may not be essential for the induction of oral tolerance [59]. Therefore, the induction of immune tolerance is a very complicated process. The process of antigen uptake, processing, and presenting to T cells by APCs, e.g. macrophage and dendritic cells, may contribute to this process, in a Treg, tolerogenic DC, and some cytokines involved mechanism.

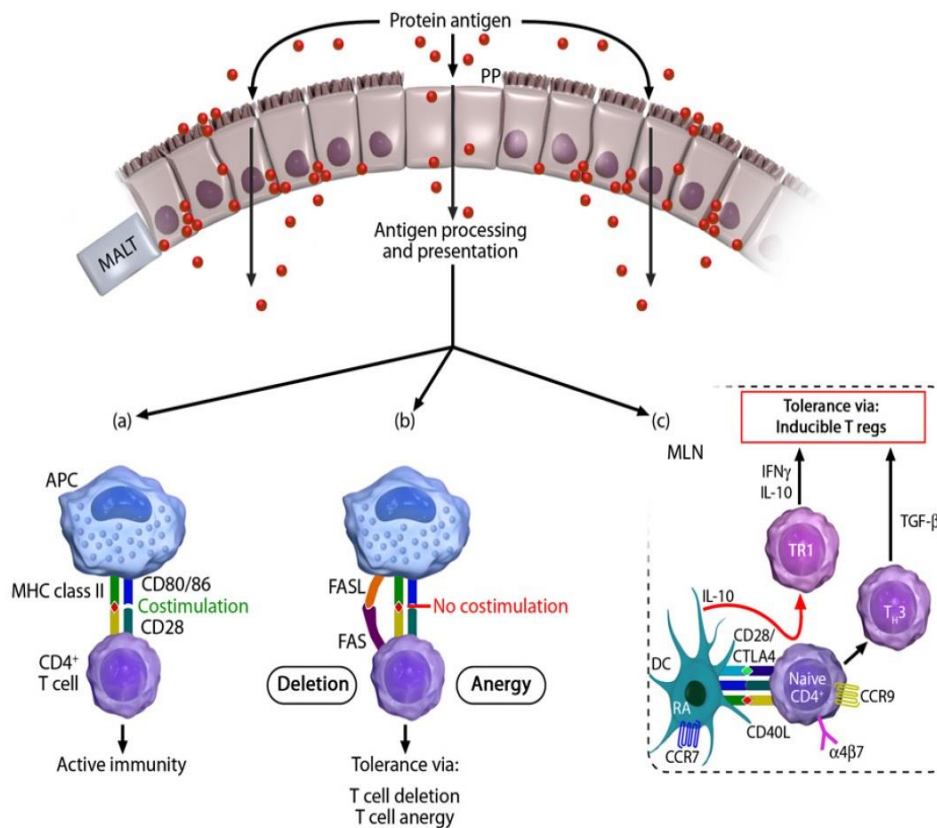


Figure 1.2 Development of immune tolerance to protein antigen (adapted from Vichery et al. [60]).

Research objectives and thesis outline

This research aimed to study the impact of extensive dry heating on the physicochemical properties of bovine whey proteins and their potential contribution to the development of immune tolerance. The first part (chapter 2 and chapter 3) of this thesis describes the changes in physicochemical properties of whey proteins after dry heating. In the second part of this thesis (chapter 4 and chapter 5), the antibody binding capacity and responses of immune cells after stimulation by extensive dry heated whey protein allergens are described. Fig. 1.3 shows the overview of the structure of this thesis.

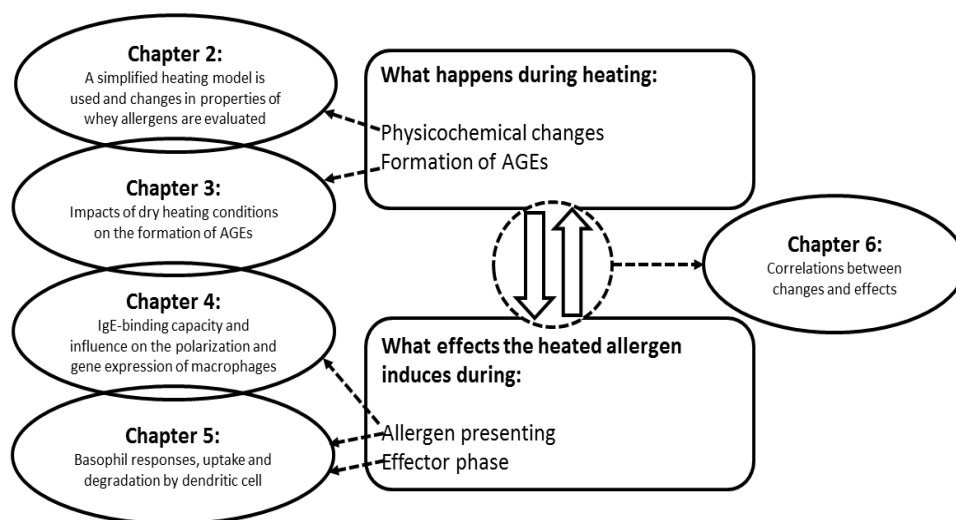


Figure 1.3 Overview of the structure of this thesis.

The thesis starts with studying the changes in the physicochemical properties upon extensive heating. A_w 0.23 and 0.59 were chosen in our studies to mimic the dry and semi-dry conditions that may occur in the preparation of muffin. Considering the important role of the pH value in the chemical reactions occurring during extensive heating, samples with pH of 5, 7, and 9 were prepared. Furosine and AGEs were used as markers of the early and advanced Maillard reaction stage, respectively. As the

main reducing sugar in milk, lactose was used in this research for the formation of Maillard reaction products. A simplified heating model is used to attempt to reproduce baking conditions. This part of the work aims to find out what happens to whey proteins during extensive dry heating. Based on these results, the responses of immune cells, which may play key roles in the induction of inflammatory or immunomodulatory responses, were studied. This was done to investigate whether the extensively heated whey proteins potentially contribute to the induction of immune tolerance on the molecular and cellular level, and these contributions result from the modifications of physicochemical properties elicited by extensive heating. These parts will contribute to answer the research question as to what modifications in the physicochemical and immunological properties of whey proteins will be induced by extensive heating. To achieve this goal, the following chapters are composed:

- **Chapter 2** studies the changes in the properties of whey proteins under controlled dry heating conditions. A simplified heating model is used to mimic the baking process. The properties of allergens that may affect their ability to induce immune responses, such as solubility, exposure of SH groups, loss of available lysine, aggregate size, and IgG-binding capacity, are studied.
- **Chapter 3** focuses on the influence of pH of the food matrix on the formation of AGEs. The formation of AGEs are tested by an sRAGE-binding assay. The gastrointestinal digestibility of extensively heated whey proteins is evaluated using an infant digestion model, and the cleavage patterns of peptides of heated proteins are compared with those of unheated proteins. In addition, the influence of digestion on the sRAGE-binding capacity of the glycated samples is studied. This part gives an overall view on the degree of Maillard reaction and its products that are produced during extensive heating at various pH values.
- **Chapter 4** evaluates the macrophage immunogenicity of glycated BLG. A correlation between the formation of AGEs and the immunogenicity is hypothesized. The IgE-binding capacity of glycated samples and their influence on the polarization and gene expression of macrophages are studied in vitro.

- **Chapter 5** continues the work on the responses of immune cells in sensitization phase food allergy due to the stimulation by glycated BLG. The uptake and degradation of glycated BLG by DCs is studied. The study on the basophil responses to glycated BLG is also included in this chapter.
- **Chapter 6** discusses the contribution of glycated whey proteins on the induction of immune tolerance based on the results of chapter 2-5. The correlation between the changes in the properties of BLG during heating and the immunological consequences is proposed. The main conclusions and recommendations for future research are presented in this chapter.

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Chapter 2

Decrease in the IgG-binding capacity of extensively dry heated whey proteins is associated with intense Maillard reaction, structural changes of the proteins and formation of RAGE-ligands



Abstract

Heat treatment is the most common way of milk processing, inducing structural changes as well as chemical modifications of milk proteins. These modifications influence the immune-reactivity and allergenicity of milk proteins. This study shows an influence of dry heating on the solubility, particle size, loss of accessible thiol and amino groups, degree of Maillard reaction, IgG-binding capacity and binding to the receptor for advanced glycation end products (RAGE) of thermally treated and glycated whey proteins. A mixture of whey proteins and lactose was dry heated at 130°C up to 20 min to mimic the baking process at two different water activities, 0.23 to mimic the heating in dry state and 0.59 for semi-dry state. The dry heating was accompanied by a loss of soluble proteins and an increase in the size of dissolved aggregates. Most of the Maillard reaction sites were found to be located in the reported conformational epitope area on whey proteins. Therefore the structural changes, including exposure of SH group, SH-SS exchange, covalent cross-links and the loss of available lysine, subsequently resulted in decreased IgG-binding capacity (up to 33%). The binding of glycation products to RAGE increased with heating time, which correlated to the stage of Maillard reaction and the decrease in the IgG-binding capacity. The RAGE-binding capacity was higher in samples with lower water activity (0.23). These results indicate that the extensive dry heating of whey proteins as occurs during baking may be of importance to the immunological properties of cow's milk allergens, both due to chemical modifications of the allergens and formation of AGEs.

Published as: Liu, F., Teodorowicz, M., van Boekel, M. A., Wichers, H. J., & Hettinga, K. A. (2016). The decrease in the IgG-binding capacity of intensively dry heated whey proteins is associated with intense Maillard reaction, structural changes of the proteins and formation of RAGE-ligands. *Food & function*, 7(1), 239-249.

Introduction

Food allergy in children is a serious health issue and an 18% increase in its prevalence from 1997 to 2007 has been reported [1]. Cow's milk (CM) is one of the major food allergies in infants and young children, affecting 2% to 3% of the general population. Fortunately, most children allergic to CM develop tolerance at an early age, although 15% to 20% have lifelong allergy [2]. However, the mechanisms behind this tolerance development and the markers to predict it are still poorly understood.

The most popular treatment option for cow's milk allergy (CMA) has long been strict elimination of the allergens, and thus all milk and milk-containing products, from the diet. As a main source of proteins, calcium, phosphorus, vitamin B12 and vitamin D, the elimination of milk from the diet likely presents nutritional disadvantages. In addition, avoiding the allergen long term, may increase the risk of an acute reaction upon the reintroduction or accidental ingestion of the allergens [3]. Recently, it was reported that most of the children can tolerate extensively heated milk, in the form of baked products [4]. The authors found that the children are more likely to become tolerant to unheated milk with addition of extensively heated milk into their diets. In comparison to other allergen-specific approaches, the addition of extensively heated milk allergens probably represents a more safe and effective approach to immunomodulation of CMA [5]. Research on the mechanism and for the establishment of safety and efficacy of adding extensive heated milk proteins into the diets of CM allergic people is ongoing. But more research needs to be done before such foods can be used for clinical practice [6].

Heat treatment is the most used process in the food industry. It induces structural changes as well as chemical modifications of milk proteins, which result in changes in the immune-reactivity and allergenicity [7]. Heat treatment of milk allergens in solution was extensively studied [8], but only a limited number of studies has been done on dry heated milk [9, 10]. Dry heating is more close to the real conditions during the production of baked products. In the study of Enomoto et al. [11], β -lactoglobulin (BLG) was dry heated with maltopentaose at 85°C. Glycation and phosphorylation slightly affected the secondary structures of BLG, and IgG-binding capacity was reduced by glycation. Besides, it was reported that the influence of dry heating and in-solution heating on structure modifications of proteins is different [12]. In some cases, heat treatment reduces the allergenicity of allergens as a result of

unfolding and aggregation [13]. The temperature applied during in-solution heating is normally too low to eliminate allergenicity of allergens, in particular when linear epitopes are involved. In the study of Sánchez-Monge et al [14], the antigenicity of BLG was reduced but not eliminated when the samples were heated in solution at 80°C to 100°C. In some baked products used in studies on the induction of immune tolerance, it was shown that during baking some parts of the material turned into a semidry or dry state, therefore reaching temperatures higher than 100°C. It was, for example, reported that technological functional properties of egg white proteins were modified as a result of formation of soluble aggregates linked with disulphide bonds and other covalent bonds when heated in such a semidry state [15]. Besides, as conformational epitopes could become both either more accessible or more damaged by heat treatment, no general conclusion can be drawn on the influence of heat treatment on the antigenicity of allergens. In some cases, the antigenicity of allergens increased after heating [16], while in some other cases it was reduced [17].

Furosine is the main stable Amadori compound in the early stage of Maillard reaction, and it is therefore considered as a good indicator of lysine damage upon heating of milk [18]. In the intermediate and late stage of Maillard reaction, so-called advanced glycation end products (AGEs) are generated as a result of the chemical reaction between reducing sugar and protein. Heat-processed foods are the main exogenous source of AGEs which may be adsorbed from gastrointestinal tract and exert its biological effects [19, 20]. Dry heating promotes the formation of dietary AGEs by more than 10- to 100- fold above the unheated state of foods [21]. These dietary AGEs increase the tissue level of AGEs and are thought to be involved in the aetiology of pathologies such as diabetes, atherosclerosis and cardiovascular disease [22, 23]. The AGEs formed during food processing may influence human physiology via the receptor for advanced glycation end products (RAGE), which has been found on most key cell types linked to an immune response [24]. Moreover, the latter studies showed that RAGE may play a role in activation of many factors connected with acute and chronic immune responses, among others the transcription factor NF- κ B and some of its downstream target genes that are well-known regulators of the adaptive and innate immune system [25]. Moghaddam et al. found that AGEs present on dry roasted prepared peanut proteins could target these proteins to antigen presenting cells [26].

However, there is no data on the influence of extensive dry heating of milk proteins on sRAGE binding properties.

Changes in IgG-binding capacity of whey proteins and formation of sRAGE binding ligands are thus expected after dry heating of whey proteins in presence of lactose. The aim of this study was therefore to investigate the effect of extensive dry heating at two different water activities, 0.23 to mimic the heating in dry state and 0.59 for semi-dry state (as measured at room temperature), on the solubility, structural changes, degree of Maillard reaction, IgG-binding capacity of whey proteins and finally the production of AGEs and RAGE-binding ligands. To provide the better molecular understanding of the finding that most of the children with CMA can tolerate extensively heated/baked milk and create a basis for studying the impact of Maillard reaction on immunogenicity of whey proteins.

Materials and methods

Materials

The spray-dried WPC that was used in this study (Wheyco GmbH, Hamburg, Germany) was reported to contain 79.8% proteins, 4.2% water, 9.5% lactose and 5.8% fat. NuPAGE 12% Bis-Tris Gels, running buffer, washing buffer and sample buffer for SDS-PAGE were provided by Life Technology (Carlsbad, USA). A molecular marker set of 9-170 kDa was obtained from Jena Bioscience (Jena, Germany). Antibodies used in ELISA analyses were provided by Abcam (Cambridge, UK). Anti-RAGE, monoclonal mouse IgG2B human RAGE antibody (MAB11451) and detection antibody were obtained from R&D systems (Minneapolis, USA). Polyclonal goat anti-mouse HRP-conjugated antibody (P0447) was obtained from DAKO (Glostrup, Denmark). All the other chemicals were from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Preparation of samples

Spray-dried WPC and lactose were dissolved in PBS pH 6.5 at a protein to lactose ratio of 1:1.5 (w/w), which is similar to the protein to lactose ratio in milk. The solution was lyophilised. Two groups of such lyophilised samples (10.6 g) in plastic sample containers were stored in desiccators for two weeks at room temperature to obtain an

a_w of 0.23 and 0.59, respectively. Powders in screw-cap test tube (Schott GL18) were heated at 130°C for time periods of 5, 10, 20 min in a heating block (Labtherm Graphit, Liebis, Germany). The samples were prepared in duplicate. Heated samples were put in an ice bath after the heat treatment to cool down immediately. Control samples (0 min) were not subjected to dry heating. Subsequently, all the powders were reconstituted in 50 ml PBS pH 7.4. After that, the solutions were centrifuged (rotor JA 25.50, Avanti Centrifuge J-26 XP, Beckman Coulter, USA) at 10,000 g for 30 min to separate the insoluble material from the soluble material. The resulting pellets were washed twice and centrifuged again. Finally, 70 mL solution was obtained for each sample. Ten mL of the dissolved sample was centrifuged again using an ultra-centrifugal device (Pall Life Science, Ann Arbor, USA) with a cut-off of 3 kDa to concentrate the samples and filter out the compounds with a molecular weight lower than 3 kDa. The dissolved protein concentration of all the samples was measured by the BCA method [27].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of conjugates

SDS-PAGE was performed under non-reducing condition. Two μ L sample was diluted in 5 μ L 4 \times concentrated sample buffer and 15 μ L MilliQ water. Then the mixture was centrifuged at 2000 rpm for 1 min and heated at 70°C in heating block (Labtherm Graphit, Liebis, Germany) for 10 min. Samples were then loaded onto 12% Bis-Tris Gels. To show the heat-induced decrease in the amount of soluble proteins, the protein concentration was not adjusted to the same level after heat treatment, but was based on a same amount of protein before heating (1 mg/mL). Gels were run at 200 V and stained with Coomassie Blue R-250, followed by destaining with washing buffer. A prestained protein marker (9 - 170 kDa) was used for molecular weight calibration. The scanned gels were analysed by Image Lab version 4.1 (Bio-Rad).

Particle sizing measurements

The particle size distribution of all the samples was measured using a Malvern Hydro 2000sm (Malvern Instruments, Malvern, UK). The determination of particle size distribution was based on the Mie scattering theory [28]. Size measurements were recorded as median diameter D0.5 and D0.9 of triplicate measurements, whereby 50%

and 90% of the particles are smaller than the size indicated, respectively. The instrument was set to measure the sample at a rate of 3000 snaps (or counts) per second. To avoid multiple scattering, the samples were added into the dispersion unit until the obscuration reached the value between 10% and 20%.

Measurement of accessible thiol groups

The quantification of accessible thiol content was performed according to the Ellman method [29]. All the samples were prepared at the same protein concentration of 1 mg/mL. Then 10 μ L of the sample was mixed well with 990 μ L 0.1 mM 5,5'-dithiobis (2-nitrobenzoate) (DTNB) reagent. Samples with only DTNB reagent were used as blank. Optical absorbance was measured at 412 nm for each sample after incubation at room temperature for 5 min.

Determination of free amino groups

The ortho-phthaldialdehyde (OPA) method was applied for quantifying free amino groups according to Nielsen et al. [30] with modifications. The dissolved protein samples were diluted to a protein concentration of 0.32 mg/mL in PBS pH 7.4. Freshly prepared OPA reagent was mixed with diluted samples in a ratio of 20:3 (v/v), followed by an incubation at room temperature for 20 min. The absorbance was measured at 340 nm against a control containing PBS and the OPA reagent. All the samples were measured in duplicate. The amount of the free amino groups in unknown samples was calculated according to a standard curve made using 0.78 to 2.5 mM L-leucine.

Determination of degree of Maillard reaction

The colour of the dry heated whey protein and lactose mixtures was measured using a ColorFlex spectrophotometer (Hunter Associates Laboratory Inc, Reston, VA). The system was calibrated using black, white and green plates. The results were recorded as L (lightness, 0=black; 100=white), a (red to green, +a=red; -a=green) and b (yellow to blue, +b=yellow; -b=blue). The colour difference index E was calculated according to the equation: $\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$ [31].

Determination of furosine was performed with an RP-HPLC system using an RP-8 furosine dedicated column (5µm, 250 × 4.6 mm, Grace, Deerfield, USA) according to the method of Resmini et al. [32] The heated and control samples were hydrolysed with 4 mL 7.95 N HCl at 110°C for 23 h in a screw-cap test tube (Schott GL18). The hydrolysates were filtered through medium-grade filter paper and transferred to new tubes. A portion of the filtrate was further purified by solid phase extraction (cartridge C18-E, phenomenex, Aschaffenburg, Germany) pre-wetted with 2 mL 0.3% acetic acid. Furosine was eluted with 3 mL 3 N HCl and collected in HPLC vials. Then furosine standard (Poly Peptide group, Strasbourg, France) and purified samples were applied to the Dionex Ultimate 3000 HPLC system with a RS Diode Array UV detector at 280 nm. The column was eluted with solvent A containing 0.4% acetic acid in MilliQ water at a flow rate of 1.2 mL/min; furosine was eluted by solvent B which contains 0.3% potassium chloride in solvent A at 22-24 min. The elution gradient was: 0-8.5 min 0% solvent B, 8.5-16.5 min multistep gradient from 0% to 50% solvent B, 16.5-19.0 min 50% solvent B, 19.0-20.5 min multistep gradient from 50% to 0% solvent B, 20.5-32.0 min 0% solvent B.

After acid hydrolysis of milk proteins, the protein-bound lactulosyl-lysine is released as 40% Lys and 32% furosine [33]. Because these products are released in a constant ratio, the amount of blocked lysine in the early stage can be calculated from the amount of furosine (mg/100 g protein) and the total lysine (mg/100 g protein) using the formula as follows [34]:

$$\% \text{ blockage due to Amadori product} = \frac{1.24 \times \text{furosine}}{\text{total lysine}} \times 100$$

Determination of IgG-binding capacity by Enzyme-Linked Immunosorbent Assay (ELISA)

Non-competitive ELISA was performed according to a method previously reported [35]. Briefly, heated and unheated whey protein samples were dissolved in coating buffer at a protein concentration of 5µg/mL. Then 100µL of these diluted samples was added to a 96 wells plate; coating buffer alone was used as negative control. The plate was incubated at 4°C overnight to coat the wells with antigen. After removal of the solution, each well was washed 3 times with 250µL of PBS-Tween (PBST). Then all the wells were saturated at 37°C for 1 h with 1% gelatin in coating buffer. Plates were

washed with PBST and 100µL of rabbit anti-whey protein IgG antibody diluted to 1:10000 in PBS pH 9.6 was added and incubated 1 h at 37°C. Peroxidase conjugated goat anti-rabbit IgG antibody diluted 1:10000 in PBS was added to each well after the plates were washed three times with PBST. Then 0.4 mg/mL o-phenylenediamine (OPD) in 50 mM citrate buffer pH 5.5 containing 0.1% (v/v) of 30% hydrogen peroxide was added to each well (100µL/well), followed by a 15 min incubation at 37°C. The reaction was stopped by addition of 50 µL 2M H₂SO₄. The absorbance was measured at 492 nm.

RAGE binding assay

The competition ELISA-based RAGE binding assay was used to measure the binding between the sRAGE receptor and dry heated samples. The specificity was tested by creating competition between a known high affinity ligand coated on the plate and the possible ligand-dry heated samples. The transparent ELISA plates (Geiner Bio-One, cat nr 655061) were coated with Glycated 90 in 1.5 mM of sodium carbonate buffer pH 9.6 overnight at 4°C. Next day the plate was washed 3 times with PBS containing 0.05% (v/v) Tween-20 and the plate was blocked with 3% BSA for 2 h at room temperature. During the blocking, the pre-incubation of samples (0.5, 5, 50, 500µg/mL) with sRAGE (recombinantly produced in E. coli. Biovendor RD172116100, 27.4 nM) was performed on polystyrene 96-well plate (Nunc). Both the sRAGE and samples were dissolved in 1.5% BSA and 0.025% Tween-20 in PBS. A positive control (maximal signal) was the sRAGE incubated without ligand in dilution buffer. Incubation was performed for 45 min at 37°C. After blocking, the coated ELISA plate was washed 3 times and then the pre-incubation mixture was added (80µL per well) followed by 1 h of incubation at 37°C. The plate was washed 3 times after which anti-RAGE antibody (monoclonal Mouse IgG2B human RAGE antibody MAB11451; 23.3 nM) was added and incubated for 30 min on the shaker at room temperature. After washing 4 times the detection antibody (polyclonal goat anti-mouse HRP-conjugated P0447; 1:1000) was added and incubated for 30 min on the shaker at room temperature. The plate was washed 4 times and TMB substrate (3,3',5,5'-tetramethylbenzidine) was added and incubated for 15 min. The colouring was stopped using 2% HCl. The plate was read using the Filtermax F5 at 450 nm with 620 nm as reference. The percentage of inhibition was determined using the follow formula: $((OD_{sRAGE} - OD_{sample}) /$

$ODsRAGE$)*100, with the average ODs of sRAGE without the competition agent as a maximal signal.

Results and discussion

Characterization of dry heated whey proteins

Analysis of PBS pH 7.4 soluble protein concentrations by the BCA method showed that the amount of soluble proteins decreased with heating time (Table 2.1). The highest decrease in protein concentration was observed between 10 and 20 min of dry heating. More than 80% of the proteins existed as insoluble aggregates after being heated for 20 min at 130°C. As expected, the amount of insoluble material increased with heating time in both groups of the samples with initial a_w of 0.23 and 0.59. Samples with a_w 0.23 showed higher loss in soluble proteins versus the samples with a_w 0.59. In the unheated samples, the soluble protein recovery reached over 97%. It has been previously shown that the denatured proteins first convert into soluble aggregates and then into insoluble aggregates during dry heating [10]. Table 2.1 indicates that the conversion into insoluble aggregates took place between 10 to 20 min of dry heating in this study.

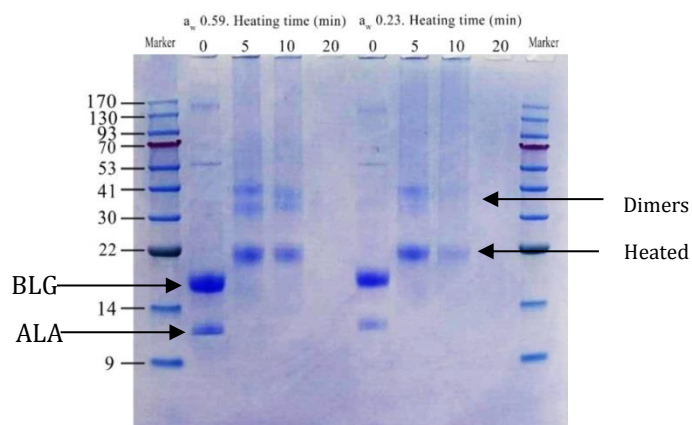


Figure 2.1. SDS-PAGE profile of dry heated whey proteins (soluble fraction at pH 7.4) under non-reducing condition. The marker with molecular weight range of 9-170 kDa was used; the samples were prepared in duplicate. Whey protein samples with water activity of 0.23 and 0.59 were heated for 5, 10, 20 min at 130°C. Non heated samples (0 min) were used as control.

SDS-PAGE under non-reducing conditions of dry heated whey proteins (Fig. 2.1) demonstrated that the amount of BLG- and α -lactalbumin (ALA)-dimers increased in the samples heated for 5 and 10 min. The electrophoretic mobility of monomeric and dimeric whey proteins decreased in heated samples, no bands with molecular weight corresponding with non-heated BLG were found for all 3 time points of heat treatment. This is in agreement with the results reported by Li et al. [36], which also showed an increase in the molecular mass of BLG and ALA. This may have resulted from the conjugation of whey protein and lactose [37]. In the samples with a_w of 0.23, the amount of soluble monomeric proteins and soluble aggregates decreased faster than that of samples with a_w of 0.59, confirming the results of protein concentration as measured by BCA (Table 2.1).

Table 2.1. Formation of insoluble material and furosine after dry heating at 130°C for different periods of time. Results are means of two independent experiments \pm standard deviation. The blocked lysine due to formation of Amadori product (%) was calculated from the amount of furosine.

Heating time (min)	Initial a_w	Soluble protein recovery	Insoluble material (g)	Furosine (mg/100g protein)	Blocked lysine (%)
0	0.23	97.9%	0.1 \pm 0.0	30.8 \pm 6.9	0.5 \pm 0.1
5	0.23	85.4%	0.6 \pm 0.3	278.3 \pm 39.5	4.5 \pm 0.4
10	0.23	67.9%*	2.7 \pm 0.8	184.8 \pm 41.8	3.0 \pm 0.5
20	0.23	11.2%*	6.4 \pm 0.1	83.2 \pm 5.5	1.4 \pm 0.3
0	0.59	97.4%	0.1 \pm 0.0	42.2 \pm 0.5	0.7 \pm 0.0
5	0.59	87.6%	0.3 \pm 0.1	294.8 \pm 19.6	4.8 \pm 0.3
10	0.59	78.4%*	1.8 \pm 0.1	241.6	2.0
20	0.59	17.7%*	6.4 \pm 0.1	109.6 \pm 5.0	1.8 \pm 0.1

* significant at $p < 0.05$

Structural consequences of dry heating on whey proteins

Particle size distribution

Laser light scattering was used to study the particle size distribution of the heated and unheated soluble samples. Soluble aggregates were formed during dry heat treatment. As shown in Fig. 2.2, no considerable changes of the mean particle size (d50) were observed in the unheated samples and samples heated for 5 and 10 min. Larger aggregates were formed in the samples that were heated for a longer time. Literature data show that for in-solution heating, a higher whey protein concentration resulted in larger aggregate size, whereas for dry state heating, formation of aggregates was mainly affected by the diffusion-controlled interactions between proteins or protein and lactose [10]. The d50 reached about 0.65 μm in samples with a_w 0.23 and 0.49 μm in samples with a_w 0.59 after 20 min heating at 130°C. The higher particle size and lower soluble protein concentration in samples with a_w 0.23 indicated that the lower a_w favours the denaturation and Maillard reaction of whey proteins. This is in line with the SDS-PAGE result, in which more soluble aggregates were observed in the heated samples with a_w 0.23 (Fig. 2.1). Aggregate size has previously been shown to increase with heating temperature, with particle sizes reaching 1 μm after heating at 148°C for 15 min. But when a whey protein solution was heated at 90°C, a mean size of only 0.13 μm was reached, even after 35 min heating [8].

Quantification of sulfhydryl groups

The amount of accessible SH groups during heat treatment depends on two competing processes: exposure of the masked SH groups and loss of accessible SH by oxidation. As shown in Fig. 2.3A, at the early stage of dry heating, the concentration of accessible SH groups increased in both groups of the samples at the a_w of 0.23 and 0.59. Thermal induced exposure of masked sulfhydryl groups probably dominated in this stage. Heating at 130°C for more than 5 min resulted in a decrease in the amount of accessible SH groups most likely due to observed formation of aggregates which may have formed by oxidation of SH. The net balance of exposure and aggregations caused the accessible SH groups to remain stable in the samples with a_w 0.23 and decrease slightly in the samples with a_w 0.59 between time points 5 and 10. The amount of

accessible SH groups increased again in the samples with a_w 0.23 after 20 min of heating.

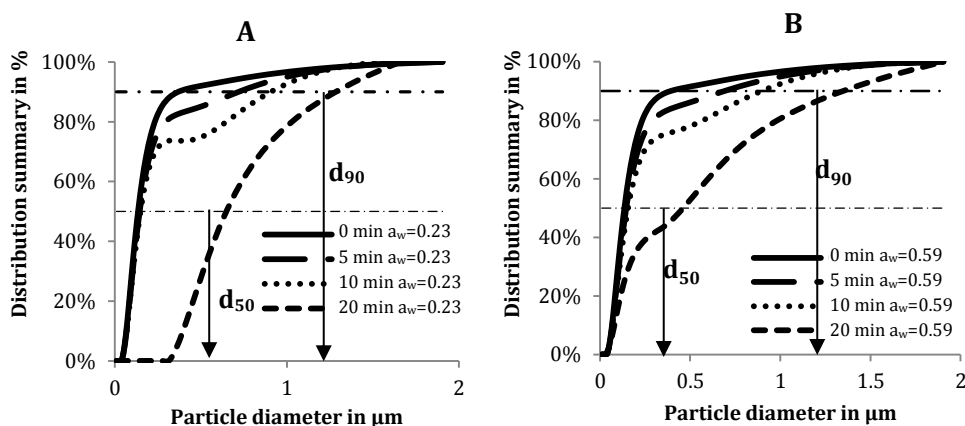


Figure 2.2. Average effects of dry heating time at 130°C on particle size distribution of soluble whey proteins, based on triplicate measurements. A: initial a_w = 0.23, B: initial a_w = 0.59.

Similar results were reported for samples heated in wet condition [38]. This indicates that with an increase in the applied heating temperature, the thiol groups become exposed as a result of molecular rearrangement of the aggregates. The exposed thiol groups interact with other molecules, small oligomers are subsequently formed through disulfide bonds. When the concentration of oligomers reaches a certain level, the so-called primary aggregates start to form.

Degree of Maillard reaction

Colour development

Some indicators of the degree of Maillard reaction, such as furosine and HMF, are colourless, and therefore cannot be reflected in the L value (lightness). The brown pigments are difficult to analyse chemically because of the complexity of their structures and low solubility due to a high molecular weight [39]. However, as an indicator of the degree of Maillard reaction, the b value (yellowness) reflects the

progress of the late-stage reaction [40]. In this study, the lightness (L) of the dry heated samples decreased upon heating for 0-10 min, due to the formation of brown pigments. After 20 min of dry heating, a slight increase in L was observed for both groups of samples with initial a_w 0.23 and 0.59. The trend of the change of lightness corroborated that of accessible amino groups. Lactose reacts with the ϵ -amino groups of whey proteins to form chromophoric, reactive side chains that eventually crosslink with other protein moieties to form insoluble brown material, which increased with heating time (Table 2.1). This may also explain the loss of accessible amino groups and the increase of soluble and insoluble coloured material. The increase in a -value also correlated with the loss of the ϵ -amino group of lysine.

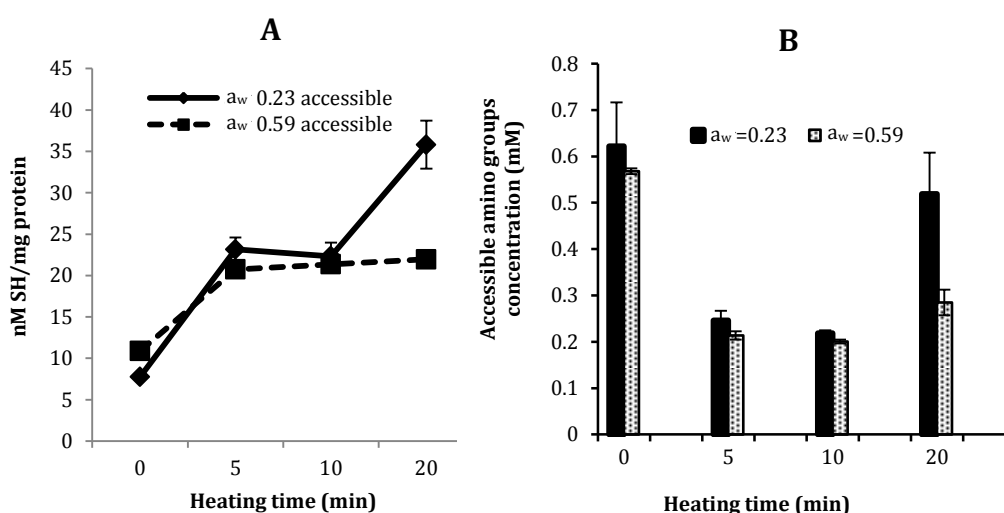


Figure 2.3. Comparison of accessible SH (A) and amino (B) groups in the samples with a_w of 0.23 and 0.59. The protein concentration of all the samples was adjusted to 0.96 mg/ml. The concentration of accessible amino groups was measured by the OPA method. Error bars represent standard deviations of duplicate measurements.

In this study, the samples were extensively dry heated. Consequently, the thermal degradation of sugars led to the formation of brown-coloured products. The amount of coloured insoluble Maillard reaction products increased with heating time (Table

2.1). This increase in insoluble coloured material may explain the increase in lightness in the soluble fraction of the samples heated for 20 min at 130°C.

Quantification of furosine and accessible amino groups

Furosine, a marker of Amadori products produced in the early stage of the Maillard reaction, was already detected in unheated samples (Table 2.1). Furosine may have been formed during the production of WPC and/or the storage of the sample for adjusting a_w . The amount of furosine increased after 5 min of dry heating at 130°C. The subsequent decrease was observed in both groups of the samples, with a_w 0.23 and 0.59 (Table 2.1). After such intense heating, the degradation of Amadori compounds in the advanced stages of the Maillard reaction would result in the subsequent decrease of furosine. The amount of furosine detected in the samples heated at different a_w correlated with the solubility of proteins after heating (Table 2.1). The formation of insoluble aggregates as an effect of advanced stages of Maillard reaction, as seen after 20 min of heating, may also explain the decrease of furosine content in these samples. The decrease in the amount of furosine in samples with a_w 0.23 was faster than that in samples with a_w 0.59, which indicates that Maillard reaction advances more easily to the advanced stage at lower a_w .

The amount of blocked lysine due to the Amadori product, which results from the reaction between the free ϵ -NH₂ group of lysine and lactose in the early stage of Maillard reaction, was calculated based on the amount of furosine (Table 2.1). However, the amount of blocked lysine originating from the advanced stages cannot be calculated from these results. The OPA method was therefore used to measure the total loss of accessible NH₂ groups, as a marker for the total loss of lysine. Fig. 2.3B showed that additional blockage takes place. Indicating that the Maillard reaction proceeded to advanced stages resulting in changes in the structure of whey proteins.

The quantity of accessible amino groups reflects the degree of glycation and aggregation of whey proteins. Probably as a result of protein aggregation, as evidenced in Fig. 2.2, the number of accessible amino groups decreased with heating time in all the samples heated for 5 and 10 min (Fig. 2.3B). There are many possible explanations for the loss of accessible amino groups. The attachment of lactose to whey proteins involves accessible NH₂ groups in the Maillard reaction between the

proteins and lactose, which leads to the formation of Amadori products. This was confirmed by the results of furosine analysis (Table 2.1). The amount of accessible amino acids increased in the samples heated at 130°C for 20 min, especially in the samples with a_w of 0.23. Similar to the explanation for result of accessible SH measurement as shown in Fig. 2.3A, this could also be the result of the unfolding and molecular rearrangement of the proteins and aggregates. In addition, the increase in the concentration of accessible NH_2 groups in the samples heated for 20 min may be explained by the breakdown of proteins and Maillard products in the advanced stage of Maillard reaction [41], as the OPA reacts readily with the N-terminal amino groups resulting from the breakdown of whey proteins and Maillard products.

IgG-binding capacity of whey proteins

ALA and BLG are major allergens in whey. The reported IgG epitopes of ALA are AA 7-18, AA 51-61 and AA 89-108 [42]. As shown in Fig. 2.4A, 67% (8 out of 12) of the lysines in ALA are in these epitopes. On BLG, AA 1-16, AA 51-64, AA 67-96 and AA 129-156 are IgG-binding epitopes. As shown in Fig. 2.4B, 80% (12 out of 15) of the lysines in BLG are located in these IgG binding areas. Consequently, the damage to lysine residues during Maillard reaction likely resulted in structural changes of epitopes on whey proteins, and may thus influence the IgG-binding capacity of whey proteins. To test this, noncompetitive ELISA was performed.

The decrease in the IgG-binding capacity of whey proteins after heating in solution was reported previously [43, 44]. These authors explained their observations by the shielding effects of epitopes owing to Maillard reaction. When the heating temperature is above 90°C, conformational epitopes may be destroyed by the aggregation between whey proteins (disulphide exchange) or by advanced Maillard reaction. On the other hand, hidden conformational epitopes on whey proteins may be exposed at temperatures higher than 65°C as a result of protein unfolding, which may coincide with exposing hidden SH groups thereby increasing the amount of accessible SH groups (Fig. 2.3A). In addition, linear epitopes may also become exposed and be involved in the aggregation of the allergen [45]. Consequently, the destruction and exposure of both conformational and linear epitopes determines the overall IgG-binding capacity of whey proteins. As shown in Fig. 2.5, the IgG-binding capacity of whey proteins decreased with heating time in both groups of samples with different

a_w . After heating at 130°C for 20 min, the IgG-binding capacity decreased to about 67% of that of the unheated samples. These results indicate that heat-induced destruction of epitopes determined the IgG-binding capacity of whey proteins in dry and semi-dry heating conditions, which is similar to what was previously only shown for proteins in a wet system [45].

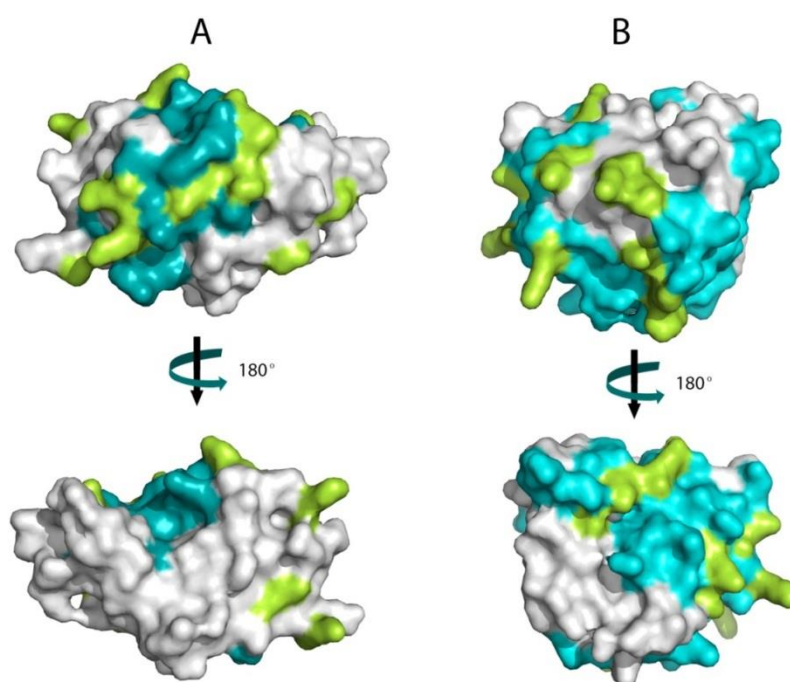


Figure 2.4. Identification of IgG epitopes and lysines on the surface of bovine β -lactoglobulin (BLG) and α -lactalbumin (ALA). The surfaces of ALA (A) and BLG (B) showing two views of the proteins, rotated 180° about the vertical axis. The conformational structures were obtained from Protein Data Bank (PDB) and displayed in PyMOL v0.99. The epitopes are coloured cyan, and the lysines are coloured Limon.

Moreover, Maillard reactions initiate changes in the hydrophilic-hydrophobic potential of heat-treated proteins [46]. As the hydrophobic properties of protein have previously been shown to play an important role in protein-protein (protein-antibody) interactions [47], the observed decrease in IgG binding may thus also result from

changes in hydrophilic/hydrophobic areas and net charge at the protein surface caused by attached sugar moieties.

Another factor that may influence IgG binding is the formation of aggregates. Spiegel reported that more compact particles are formed at temperatures above 90°C in wet condition [48]. Antibodies can only reach the epitopes on the surface of these particles [8]. As a result, the IgG-binding capacity decreases with heating time and hence particle size (Fig. 2.5B). In addition, the epitopes on the surface may simultaneously be destroyed in two ways: by aggregation of proteins and by Maillard reaction with lactose. This may result in the loss of accessibility of some epitopes initially located on the surface of whey proteins (Fig. 2.4). Subsequently, the IgG-binding capacity of the PBS soluble allergens decreases with the increase of particle size (Fig. 2.5B). Similar results were reported on peanut allergen Ara h 1 and hazelnut allergen Cor a 11 [49]. A significant decrease in IgE binding capacity of Ara h 1 and Cor a 11 roasted with glucose was observed, which was accompanied by an increase in their particle size.

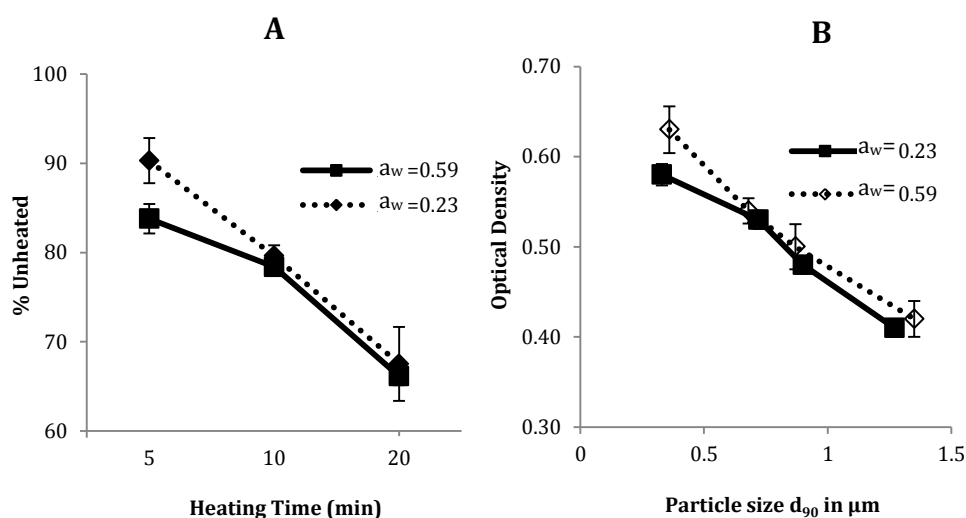


Figure 2.5. IgG-binding capacity of heated whey proteins (A) and its correlation with particle size (B). The IgG-binding capacity is expressed as the percentage of signal obtained by noncompetitive ELISA on unheated whey protein samples. Error bars represent standard deviations of triplicate measurements.

Formation of RAGE-binding ligands

The binding between sRAGE and its ligand was dose-dependently inhibited by glycated whey protein samples (Fig. 2.6A and Fig. 2.6B). As presented in Fig. 2.6C and Fig. 2.6D, unheated sample is not able to inhibit RAGE-ligand binding while up to 90% of inhibition was observed for dry-heated whey proteins. After 10 min of dry heating for the concentration of 50 µg/ml, the ability of heated whey proteins (both a_w 0.23 and 0.59) to bind to RAGE was higher than that of the positive control samples (amyloid beta). The data suggest that more RAGE binding ligands were formed in the samples with a_w 0.23 or the formed Maillard reaction products/aggregates have higher RAGE binding potential than products of heating with a_w 0.59. Furosine was already detected in the unheated samples due to either the production process of WPC or the storage of the samples at room temperature for a_w adjustment (Table 2.1); however, the unheated sample did not show any RAGE binding activity, indicating that no advanced Maillard reaction had yet occurred. Less PBS-soluble proteins, as shown in Fig. 2.1, and larger aggregates in samples with a_w 0.23 (Fig. 2.2) indicate that extensive dry heating at a_w 0.23 favoured the aggregation and Maillard reaction of the samples, which correlated with the sRAGE-binding results that showed more ligands were present in the sample heated at this water activity. In humans, there are two types of RAGE receptors, a soluble form (sRAGE) and a membrane-bound form. In this study, the ELISA based RAGE-binding assay was performed to analyse the binding ability of BLG-derived Maillard reaction products to the sRAGE. The low level of this form of the receptor are normally present in the human plasma and may bound the food derived AGEs which have been absorbed in the gut and got to the bloodstream. Compared to the interactions between AGEs and cell surface RAGE, the binding of sRAGE and AGEs offers protective effects as AGE inhibitors [50]. Two distinct mechanisms are known for the generation of the soluble form of the RAGE receptor. First, alternative splicing can lead to removal of the transmembrane or cytosolic domains of membrane form of receptor. Secondly, metalloproteases ADAM10 and MMP9 are able to cleave the extracellular domain of membrane form of RAGE. Both mechanisms can form the sRAGE that binds ligands with similar affinity as the membrane bound receptor [25, 51, 52].

The interaction between RAGE and the AGEs may be one of the mechanisms explaining the immunomodulatory properties of AGEs. Two unrelated studies showed the binding between RAGE and AGEs on antigen presenting cells. Hilmenyuk and co-workers found that immature dendritic cells (DC) express RAGE stronger compared to mature DCs. Moreover, an enhanced expression of RAGE on the immature DCs was observed after exposure to AGEs [53]. In a recent study, Mueller and others determined the binding of Maillard reaction products present on raw peanut and oven-roasted peanut towards IgE and RAGE. The authors showed that RAGE interact with the AGE modified recombinant peanut allergen Ara h1 and not with the unmodified recombinant Ara h1 [54]. These outcomes are in line with our findings. We did not observe an interaction of unheated whey proteins with sRAGE. The sRAGE binding properties were increasing with the time of heating and the formation of aggregates what suggest that the aggregated proteins have a higher binding properties to sRAGE what may lead to an increased uptake of glycated proteins/agglomerated by dendritic cells.

In the early stage of Maillard reaction, the moderate glycation has only slight effects on the allergenicity of BLG, whereas after the formation of AGEs in the advanced stage, a clear “masking” effect on the reaction between the allergens and IgE antibodies was observed [35]. This effect, and the formation of new epitopes [55], determine the immunogenicity of glycated allergens. We showed the decreased IgG binding to dry-heated whey proteins, however, the formed Maillard reaction products showed high affinity to RAGE receptor, which may influence that immune response. Besides, the AGEs can also affect the immunogenicity of allergens via activation of key immune cells such as macrophages and dendritic cells [56].

As heat-treated foods are considered as the main exogenous source of AGEs, the preparation method has attracted serious attention especially in the preparation of infant nutrition. Previous studies have reported that water content, temperature, heating time and pH are related to the formation of AGEs [21, 57]. In this study, moisture (a_w) and heating time were both found to significantly affect the production of AGEs and also the formation of sRAGE-binding ligands.

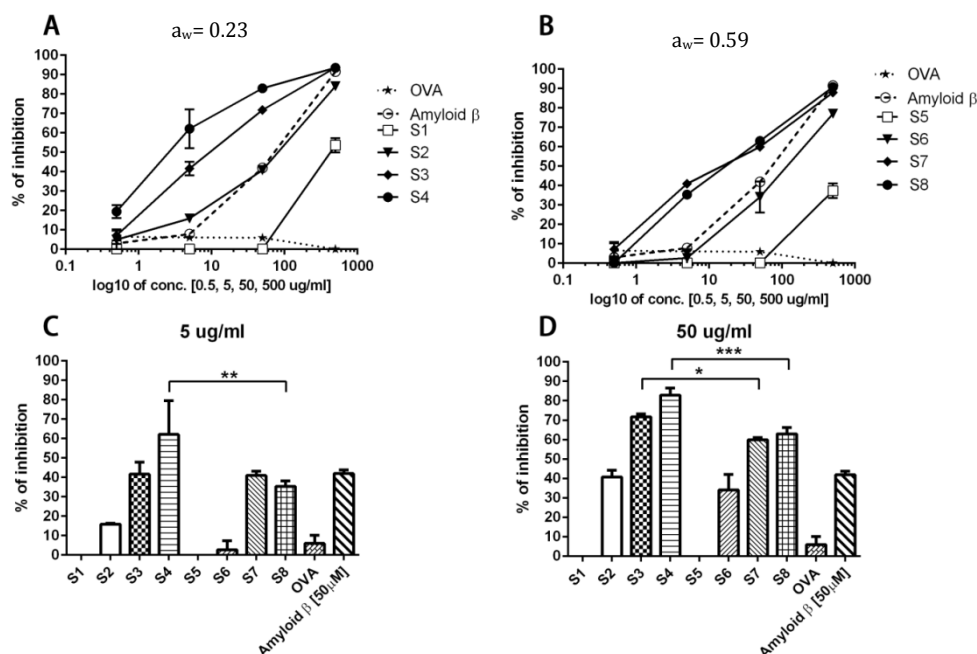


Figure 2.6. Inhibition capacity of dry heated samples on RAGE binding. The percentage of inhibition of RAGE binding is presented as a function of the protein concentration of samples with a_w 0.23 (A) and 0.59 (B). The influence of a_w on the formation of AGEs was evaluated in the samples with protein concentration 5 $\mu\text{g/ml}$ (C) and 50 $\mu\text{g/ml}$ (D). s1, s5: unheated; s2, s6: dry heated for 5 min; s3, s7: dry heated for 10 min; s4, s8: dry heated for 20 min; s1-s4 a_w 0.23; s5-s8 a_w 0.59. OVA was used as negative control. Amyloid β was used as positive control. Data are expressed as mean \pm SD from three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Conclusion

Extensive dry heating at lower a_w (0.23) favours the aggregation and Maillard reaction of the samples. Unfolding and aggregation of the whey proteins determined the accessibility of the thiol groups and amino acids, which also correlated to the stage of Maillard reaction. As most of the lysine residues are located in the epitope areas of whey proteins, the Maillard reaction between these lysine residues and lactose will have resulted in modifications of the conformational IgG epitopes of whey proteins.

The IgG-binding capacity of soluble allergens subsequently decreased during dry heating.

The IgG-binding capacity of the samples heated for 20 min at 130°C is still 67% of that of unheated proteins. The fact that extensively heated milk can be consumed by most cow's milk allergic people and accelerate the resolution of CMA is thus probably not only due to a decrease in the IgG-binding capacity of soluble allergens. The large portion of the remaining insoluble proteins, may be also responsible for the induction of immune tolerance to CM allergens, due to the modifications these allergens underwent.

Dry heating promotes the formation of sRAGE-binding ligands, dependent on heating conditions (water activity and heating intensity). The sRAGE-binding capacity correlated positively with the formation of aggregates but negatively with IgG-binding capacity of the extensively dry-heated samples.

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Chapter 3

Generation of soluble advanced glycation end products receptor (sRAGE)-binding ligands during extensive heat treatment of whey protein/lactose mixtures is dependent on glycation and aggregation



Abstract

Heating of protein- and sugar-containing materials is considered the primary factor affecting the formation of advanced glycation end products (AGEs). This study aimed to investigate the influence of heating conditions, digestion, and aggregation on the binding capacity of AGEs to the soluble AGE receptor (sRAGE). Samples consisting of mixtures of whey protein and lactose were heated at 130°C. An in vitro infant digestion model was used to study the influence of heat treatment on the digestibility of whey proteins. The amount of sRAGE-binding ligands before and after digestion was measured by an sRAGE-binding assay. Water activity did not significantly affect the extent of digestibility of whey proteins dry heated at pH 5 (ranging from 3.3 ± 0.2 to $3.6 \pm 0.1\%$ for gastric digestion and from 53.5 ± 1.5 to $64.7 \pm 1.1\%$ for duodenal digestion), but there were differences in cleavage patterns of peptides among the samples heated at different pH values. Formation of sRAGE-binding ligands depended on the formation of aggregates and was limited in the samples heated at pH 5. Moreover, the sRAGE-binding activity of digested sample was changed by protease degradation and correlated with the digestibility of samples. In conclusion, generation of sRAGE-binding ligands during extensive heat treatment of whey protein/lactose mixtures is limited in acidic heating condition and dependent on glycation and aggregation.

Published as: Liu, F., Teodorowicz, M., Wichers, H. J., Van Boekel, M. A., & Hettinga, K. A. (2016). Generation of sRAGE-binding ligands during extensive heat treatment of whey protein/lactose mixtures is dependent on glycation and aggregation. *Journal of Agricultural and Food Chemistry*, 64 (33), pp 6477–6486

Introduction

Advanced glycation end products (AGEs) can be generated from nonenzymatic glycation and oxidation of proteins. AGEs constitute a group of heterogeneous compounds (e.g. pentosidine, carboxymethyllysine (CML), imidazolone, pyrralin, GA-pyridine, etc.) [1]. They occur both in exogenous sources and slowly in biological systems in vivo, where they contributes to tissue modifications [2]. The exogenous source is mostly related to heat-processed food products that are rich in proteins and reducing sugars. These heat-processed foods, such as extensively heated milk and egg products, are intensely studied in clinical research on the resolution of allergy [3, 4]. However, due to the possible involvement of AGEs in the etiology of several diseases, there is a need to optimize the heating conditions to limit the generation of AGEs during the preparation of such food products.

The conditions during heat treatment, including nutrient composition, temperature, heating time, amount of moisture, and pH of the food matrix, affect the generation of AGEs in foods during heating [5]. Highest AGE levels were found in high-temperature processed nut and grain products [6], which are both rich in proteins and reducing sugars. In the thermal processing of proteins and reducing sugars, such as in the baking process of muffins or cookies, a substantial amount of AGEs may form. After the spray-drying of infant formulas, the amount of AGEs may exceed those present in human breast milk up to 670-fold [7]. Another important factor that affects the generation of AGEs is the pH of the food matrix. Heat treatment of food products in acidic environment was recommended to limit the AGE formation [8]. The products and pathways of the Maillard reaction are influenced by the initial pH of the solution with reactants and buffering capacity of the system. H^+ ions are required to catalyze both the Amadori and Heyns rearrangements. The degradation of Amadori compounds is also affected by pH [9]. To inhibit the formation of AGEs, apart from controlling the heating conditions during thermal processing, also various inhibitors have been proposed in the past few years [10]. It is reported that such synthetic compounds are responsible for some adverse effects in in vivo tests [11]. Therefore, instead of synthetic compounds, several natural products, such as polyphenols and flavonoid-rich extracts that have AGE inhibitory effects because of their antioxidant properties, have been extracted from green tea, mung bean, and grapes. However, the extracting efficiency needs to be improved before industrial applications.

Alternatively, adjustment of the pH of the food matrix represents a simpler, more effective, and economical way to inhibit the formation of AGEs [12, 13].

Before the AGEs or AGE-induced peptides reach the systemic circulation, gastrointestinal (GI) digestion could affect their bioavailability and metabolic fate. Faist et al. reported that a large amount of advanced Maillard reaction products (MRPs) could be recovered in urine (16% of the consumed amount) and feces (40-50% of the consumed amount) in rats [14]. Similarly, most of the dietary pyrroline is absorbed and excreted through urine within 48 h [15]. Low molecular weight (LMW) AGEs take up a large portion of excreted AGEs [16], indicating a high excretion rate of LMW AGEs and a comparatively short time in which they interact with functional proteins in the body of healthy individuals. Compared to LMW AGEs, larger fragments of nonexcreted AGEs bind nonspecifically to proteins in the body, especially in the liver and kidney. These larger fragments have comparatively higher affinity for proteins, leading to extensive tissue retention, which would subsequently function in the development of disease [16]. In terms of the digestion of proteins, prior glycation altered the peptide profiles after digestion. Some of these peptides formed after digestion may possess beneficial biological properties, such as antimicrobial, antihypertensive, and immunomodulatory activities [17]. Depending on the structural features of the protein, Maillard reaction may either increase [18] or decrease [19] the in vitro protein digestibility. These effects induced by the Maillard reaction may result from the reduction in the availability of lysine, structural changes in glycosylated proteins, or even inhibited protease activity by MRPs [20].

In our previous work, we found that sRAGE-binding ligands were formed during extensive heating, especially in samples with lower water activity ($a_w = 0.23$), which is the ratio of the vapor pressure of water in a food divided by the vapor pressure of pure water at the same temperature [21]. The present work aims at studying the feasibility of using controlled pH and a_w to obtain extensively heated whey proteins containing limited amount of sRAGE-binding ligands. pH 5 and 7 were chosen to cover the diversity of commercial whey products [22]; the range was extended to pH 9 because a high pH is known to change the protein unfolding process, certainly in combination with high temperature. $a_w = 0.23$ was chosen to mimic heating in the dry state and $a_w = 0.59$ for semidry state, which are the water activities that reflect the baking process, which has been used in tolerance induction studies [4]. An infant GI

digestion model was employed to study the digestive fate of AGEs. The amount of sRAGE-binding ligands before and after digestion was measured by an ELISA-based sRAGE assay. The peptide profile was investigated by HPLC to analyse the influence of heating conditions on proteolytic breakdown of the glycated whey proteins. To investigate the influence of aggregation on the sRAGE-binding capacity of glycated proteins, the size of aggregates was measured by size exclusion chromatography.

Materials and methods

Materials

The spray-dried WPC that was used in this study (Wheyco GmbH, Hamburg, Germany) was reported to contain 79.8% proteins, 4.2% water, 9.5% lactose, and 5.8% fat. NuPAGE 12% Bis-Tris Gels, running buffer, washing buffer (20% methanol, 10% acetic acid, and 70% demineralized water), and sample buffer containing lithium dodecyl sulphate at a pH of 8.4 for SDS-PAGE were provided by Life Technology (Carlsbad, CA, USA). A molecular marker set of 5-245 kDa was obtained from Jena Bioscience (Jena, Germany). Pepsin (P6887), pancreatin (P1750), bile extract porcine (B8631), and Pefabloc (76307) were provided by Sigma (St Louis, MO, USA). β -lactoglobulin (L3908) (BLG) was purchased from Sigma (Zwijndrecht, The Netherlands). All other chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Preparation of samples

Spray-dried WPC and lactose were dissolved in PBS at pH 5, 7, and 9 at a protein to lactose ratio of 1:1.5 (w/w), which is similar to the protein to lactose ratio in milk. The solutions were lyophilised. Three groups of such lyophilised samples (2.5 g) in plastic sample containers were stored in desiccators with saturated potassium acetate, sodium bromide, and ammonium chloride solution for 2 weeks at room temperature to obtain an a_w of 0.23, 0.59, and 0.79, respectively. Powders in screw-cap test tubes (Schott GL18) were heated at 130°C for 10 min in a heating block (Labtherm Graphit, Liebis, Germany). As the Maillard reaction may take place, even if at a largely limited rate, during the two-week storage at room temperature for adjusting a_w , an unheated whey protein/lactose mixture without adjusted a_w was used as control. The

samples were prepared in duplicate. Heated samples were put in an ice bath after the heat treatment to cool immediately. Subsequently, all of the powders were reconstituted in 30 mL of PBS (pH 7.4). After that, the solutions were centrifuged (rotor JA 25.50, Avanti Centrifuge J-26 XP, Beckman Coulter, Indianapolis, IN, USA) at 10000 g for 30 min to separate insoluble from soluble material. Lactose and salts in the samples were filtered out using an ultracentrifugal device (Pall Life Science, Ann Arbor, MI, USA) with a molecular weight cutoff of 3 kDa. The amount of remaining accessible amino groups in PBS-soluble protein was measured using the ortho-phthalaldehyde (OPA) method according to Nielsen et al. [23]. The pellet was lyophilised again for 24 h and then the weight was measured. Protein content of the dried pellet was also measured by the Dumas (Thermo Quest NA 2100 Nitrogen and Protein Analyser, Interscience, Breda, The Netherlands), which measures the protein content by converting all nitrogen forms in the sample to nitrogen oxides through combustion at 800-1000°C. In this study, a nitrogen to protein conversion factor 6.38 was chosen as recommended for milk and milk products [24].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of conjugates

SDS-PAGE was performed under nonreducing condition. A 2 µL sample with a protein concentration of 2 mg/mL was diluted in 5 µL of 4 × concentrated sample buffer and 15 µL of MilliQ water. Then the mixture was centrifuged at 2000 rpm for 1 min and heated at 70°C in a heating block (Labtherm Graphit, Liebisch, Germany) for 10 min. Samples were then loaded onto 12% Bis-Tris gels. Gels were run at 200 V and stained with Coomassie Blue R-250 0.1% (w/w), followed by destaining with washing buffer. A prestained protein marker (5-245 kDa) was used for molecular weight calibration. The scanned gels were analysed by Image Lab version 4.1 (Bio-Rad).

In vitro gastrointestinal digestion

The digestion was performed following the methods previously reported [25]. The following modifications were done to specifically mimic infant GI digestion [26]: the pepsin concentration was decreased by a factor of 8; the pancreatin concentration was decreased by a factor of 10; the amount of pancreatin inhibitor was reduced by a factor of 10 accordingly; the bile salts concentration was decreased by a factor of 4.

Briefly, 30 mL of the reconstituted sample with protein concentration of 32.7 mg/mL was mixed well with 22.5 mL of simulated gastric fluid (SGF [25]). Porcine pepsin was added to reach an enzyme activity of 61.25 U/mg protein in SGF. After that, 15 µL of 0.3 M CaCl₂ was added, followed by an adjustment of the pH to 3 with 0.2 M HCl. The mixture was then incubated at 37°C for 2 h in a shaking water bath. The enzymatic hydrolysis was stopped by adjusting the pH to 7 and subsequent snap-freezing in liquid nitrogen.

In duodenal digestion, 20 mL of gastric digesta was mixed with 11 mL of simulated intestinal fluid (SIF) [25] electrolyte stock solution. Porcine pancreatin (4 USP specifications) was added to reach an enzyme activity of 200 U/mL as measured by the method of Vandermeers et al. [27], followed by the addition of 2.5 mL of freshly prepared bile salts at a concentration of 160 mM as measured following the method reported by Collins et al. [28]. After that, 40 µL of 0.3 M CaCl₂ was added. The pH of the chyme was adjusted to 7 with 1 M HCl. After incubation at 37°C for 2 h in a shaking water bath, the enzyme was inactivated by the addition of the protease inhibitor Pefabloc SC to reach a concentration of 1 mM in the duodenal chyme.

The digestibility of the samples was measured by the formation of amino groups detected by the OPA method according to Nielsen et al. with modifications [23]. The dissolved protein samples were diluted to a protein concentration of 0.82 mg/mL in PBS (pH 7.4). Then 26 mg of L-leucine was dissolved in 10 mL of Milli-Q water, which corresponds to 20 mM L-leucine. A sequence of samples with L-leucine concentrations of 0, 0.156, 0.625, 1.25, and 2.5 mM were used to obtain a standard curve. Freshly prepared OPA reagent (0.1 M sodium tetraborate, 3.5 mM SDS, 6.0 mM OPA, and 5.7 mM DTT) was mixed with diluted samples in a ratio of 20:3 (v/v), followed by incubation at room temperature for 20 min. The absorbance was measured at 340 nm against a control containing PBS and the OPA reagent. All of the samples were measured in triplicate. The amount of free amino groups in unknown samples was calculated according to the standard curve. The calculation of the degree of enzymatic hydrolysis (DH) was according to the formula

$$DH = h / h_{\text{tot}} * 100\%$$

where h_{tot} is the number of peptide bonds in equivalents/g protein and was estimated following the method of Adler-Nissen et al.; the h_{tot} used for whey proteins in this

study was 8.8 [29]. The value of h was obtained from the OPA method as mequiv L-leucine NH_2/g protein.

RP-HPLC of duodenal digesta

The duodenal digesta were centrifuged at 10000 g for 10 min. The supernatant was collected and filtered through a 0.45 μm syringe filter. Ten microliters of the filtered samples was loaded onto the Aeris PEPTIDE 3.6u XB-C18 250 \times 4.6 mm column attached to a Dionex Ultimate 3000 HPLC system with RS Diode Array UV detector operating at 204 nm. The column was eluted using 0.1% formic acid in Milli-Q water as solvent A and 0.1% formic acid in acetonitrile as solvent B. The elution gradient was: 0-3 min 3% solvent B, 3-33 min gradient from 3% to 65% solvent B, 33-38 min gradient from 65% to 3% solvent B, 38-41 min 3% solvent B. All the samples were eluted with a flow rate of 1.2 mL/min.

Size exclusion chromatography (SEC)

Separation by size of glycated whey protein aggregates was carried out by SEC on an AKTA micro system (GE Healthcare, Uppsala, Sweden) equipped with a Superdex 75 HR 10/300 column (GE Healthcare) as described by Butré et al [30]. The experiments were performed at 20°C with 10 mM sodium phosphate buffer with 150 mM NaCl at pH 8.0 (filtered through a 0.2 μm membrane), and a flow rate of 800 $\mu\text{L}/\text{min}$. The elution of the injected samples (50 μL) was monitored at 280 nm. Ovalbumin (45 kDa), BLG (18.4 kDa), bovine serum albumin (69 kDa), and α -lactalbumin (14.1 kDa) were used as standards.

Circular dichroism (CD)

BLG and lactose were dissolved in PBS pH 7.4 at a protein to lactose ratio of 1:1.5 (w/w) to study the influence of dry heating on the secondary structures of BLG. The solution was lyophilised. Two groups of such lyophilised samples in plastic sample containers were stored in desiccators for two weeks at room temperature to obtain an a_w of 0.23. Powders in screw-cap test tube (Schott GL18) were heated at 130°C for time periods of 0, 2, 4, 6 and 8 min in a heating block (Labtherm Graphit, Liebisch, Germany). Far-UV CD spectra were recorded at 20°C using a J-715 spectropolarimeter (JASCO) equipped with a temperature controlled cuvette holder. The CD-spectra,

ranging from 185 nm to 260 nm, of each sample was acquired using a 1 mm quartz cuvette (Starna, type 21) at a scan speed of 100 nm/min. Each sample was diluted to a protein concentration of 0.2 mg/ml using 10 mM potassium phosphate buffer pH 6.9 before the measurement. The far-UV CD-spectra of 10 mM potassium phosphate buffer pH 6.9 was acquired to correct the baseline.

sRAGE binding assay

The competition ELISA-based RAGE binding assay was used to measure the binding between the soluble form of RAGE receptor and the heated WPC, both before and after digestion. The specificity was tested by creating competition between a known high affinity ligand coated on the plate and the possible ligand - dry heated WPC samples. The transparent ELISA plates (Geiner Bio-One, cat nr 655061) were coated with glycated 90 (soy protein extract glycated with glucose for 90 minutes), which showed highest binding capacity to human sRAGE receptor as measured by direct ELISA assay, in 1.5 mM of sodium carbonate buffer pH 9.6 overnight at 4°C. Next day the plate was washed with PBS containing 0.05% (v/v) Tween-20 and the plate was blocked with 3% BSA for 1 h at room temperature. WPC samples in the number of dilutions (0.25, 2.5, 25, 250, 2500 µg/mL) were pre-incubated (45 min, 37°C) with sRAGE (recombinantly produced in *E. coli*. Biovendor RD172116100, 1 µg/mL) in the dilution buffer (1.5% BSA and 0.025% Tween-20 in PBS). After blocking, the coated ELISA plate was washed and then the pre-incubation mixture was added followed by 1 h of incubation at 37°C. The plate was washed and anti-RAGE antibody (monoclonal mouse IgG2B human RAGE antibody MAB11451; 1 µg/mL) was added and incubated for 30 min on the shaker at room temperature. After washing the detection antibody (polyclonal goat anti-mouse HRP-conjugated P0447; 1:1000) was added and incubated for 30 min on the shaker at room temperature. The plate was washed and TMB substrate (3,3',5,5'-tetramethylbenzidine) was added and incubated for 15 min. The colouring was stopped using 2% HCl. The plate was read using the Filtermax F5 at 450 nm with 620 nm as reference. The percentage of inhibition was calculated using the following formula: $((OD_{sRAGE} - OD_{sample}) / OD_{sRAGE}) * 100$, where OD sRAGE was the signal obtained for sRAGE incubated without the competition agent (WPC sample) in the dilution buffer (maximal signal). All tests were performed two times in triplicate.

Statistics

Two independent experiments for two batches of treated WPI/lactose samples were performed in duplicate or triplicate. Data were expressed as mean \pm SD. Two-way ANOVA was used to determine the statistical significance of the data. Significance was defined at $p < 0.05$. Statistic calculations were performed by GraphPad Prism 4 software.

Results and discussion

Soluble protein recovery

As shown in Fig. 3.1, the solubility of the whey proteins after heat treatment was affected by pH of the sample before heating. In the samples with a_w of 0.23, the solubility was slightly affected by pH. Comparing the samples with a_w 0.79 and a_w 0.59 prepared at pH 9 with that at pH 5, a significant loss in the soluble protein recovery was observed (Fig. 3.1A). At all pH values, heat treatment of samples at a higher water activity yielded more insoluble proteins (Fig. 3.1B). This corresponds with the study of Plancken et al., which showed that dry heating of egg proteins did not induce a significant loss of protein solubility in samples, whereas at higher moisture content, protein solubility was decreased [31]. Regarding the influence of pH on the solubility of glycated proteins, Gulzar et al. studied the dry heating (a_w 0.23) of whey proteins at pH 2.5, 4.5, and 6.5, and observed a greater amount of insoluble aggregates in samples with higher pH [22].

In addition, protein glycation that occurs between lysine and reducing sugars also largely affects the solubility of proteins [32]. Lysine was moderately lost below pH 8.0 in both early and advanced glycation stage, and the loss increased with increasing pH [12], indicating that protein glycation increased with increasing pH. In alkaline conditions, besides glycation also caramelization reactions were greatly accelerated [33], both of which account for the formation of insoluble precipitate.

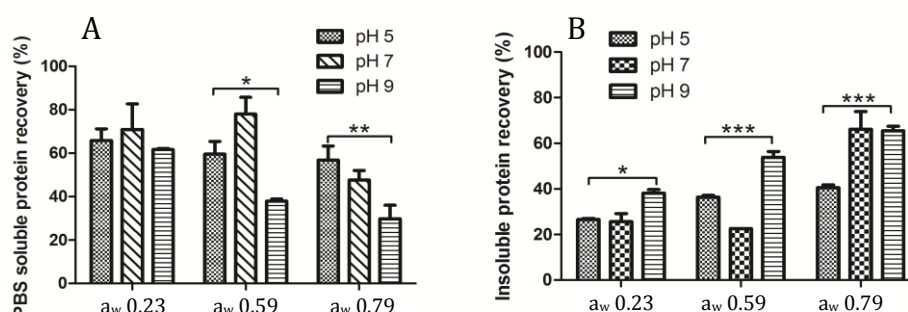


Figure 3.1. Solubility of whey proteins after heat treatment with lactose at 130°C for 10 min as a function of initial a_w . Protein content in PBS pH 7.4 supernatant (A) and pellet (B) was measured by the Dumas method. Data are representative of two independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Heating-induced aggregation of whey proteins at different pH

After heat treatment, the amount of remaining accessible amino groups in PBS-soluble protein was measured using the OPA method. When comparing the samples with the same water activity, OPA reactivity increased with increasing pH (Fig. 3.2A). This seems in conflict with the results shown in Fig. 3.1, which shows that less aggregate was generated in the samples prepared at pH 5, in which a lower glycation-induced decrease in the accessibility of amino groups would be expected. Both the unfolding and glycation of proteins can affect the amount of accessible amino acid. Thermal-induced unfolding of the protein exposes the free amino groups originally buried in the interior of the protein, whereas glycation reduces the accessibility of amino groups by reacting with reducing sugars. These two competing processes determine the final amount of accessible amino groups. In this study, the OPA results suggest that the level of unfolding of proteins was lower in the samples prepared at lower pH (pH 5) and thereby decreased the final accessibility of amino groups. BLG, one of the main whey proteins, was shown to have an increased stability at acidic pH [34]. The lower level of unfolding of the protein at acidic condition was also reported in the study of Belloque et al. [35]. In the presence of α -lactalbumin, BLG also retains its enhanced thermal stability at low pH [36]. Consequently, the amount of accessible amino acid is lower at lower pH due to the lower level of unfolding of whey proteins.

SDS-PAGE analysis of glycosylated whey proteins was performed. As expected, the density of the BLG monomer band in all a_w groups decreased with increased pH during heating, whereas the density of the band of higher molecular weight aggregates not entering the gel increased (Fig. 3.2B). This is in agreement with the soluble protein recovery results as shown in Fig. 3.1. These results indicate that unfolding, denaturation, and aggregation are all limited in the samples heated at pH 5.

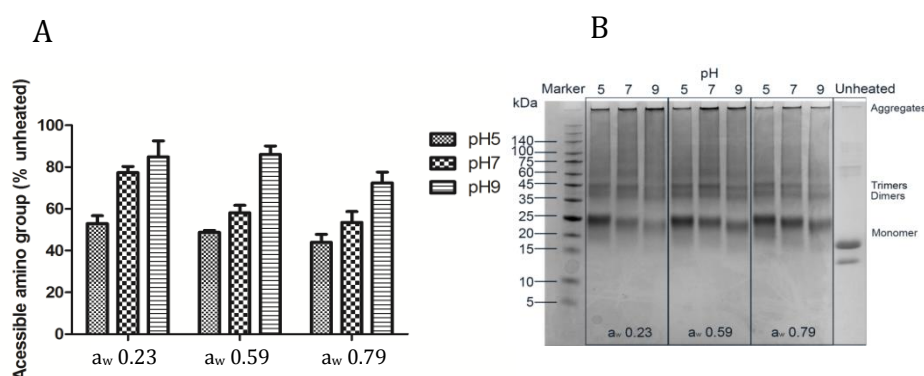


Figure 3.2. Influence of dry heating on the content of accessible amino groups (A) and denaturation (B) of PBS-soluble whey proteins. Data are representative of two independent experiments.

Gastrointestinal (GI) digestion of the extensively heated whey proteins

As cow's milk allergy (CMA) mainly affects infants and young children, an infant digestion model is normally applied in the study of CMA. Compared to an adult digestion model, the levels of protease, phosphatidylcholine and bile salts are reduced in an infant digestion model [26], and the infant gastric fasting pH (2.5) is suboptimal for pepsin, which may result in the incomplete digestion of protein allergens accounting for the subsequent inappropriate immune responses. The mixture of supernatant and PBS insoluble material was subjected to in vitro GI digestion. As shown in Fig. 3.3A, the gastric digestibility of the samples with a_w 0.23 and 0.59 was significantly lower in samples prepared at pH 5 than that at pH 7. In the group of samples with a_w 0.79, the gastric digestibility decreased with increased pH. The explanation for this may be that unfolding of the protein exposes the hydrophobic

amino acids to the proteolytic enzymatic action, thereby increasing digestibility [37]. However, the reaction between amino groups and carbohydrates and intramolecular reaction between amino acids within the protein molecule retard the enzymatic hydrolysis [38]. The final digestibility of the heated protein samples is determined by both the digestibility of PBS soluble protein, which is mainly affected by protein unfolding, and the digestibility of PBS-insoluble proteins as reflected by the amount of precipitation. Based on the results of our study, the solubility of the proteins is more important than the unfolding in determining the final gastric digestibility, as shown by the positive correlation between the gastric digestibility (Fig. 3.3A) and the solubility (Fig. 3.1A) of the whey proteins.

With regard to duodenal digestion, heat treatment at different water activity and pH conditions did not result in a significant difference in digestibility of whey proteins, except for the samples with a_w 0.59 and a_w 0.23 at pH 9 (Fig. 3.3B). High resistance to GI digestion of the thermal-induced precipitate in this sample may be responsible for the lower digestibility, with 90% of the precipitate in the samples with a_w 0.59 was still insoluble after duodenal digestion (data not shown).

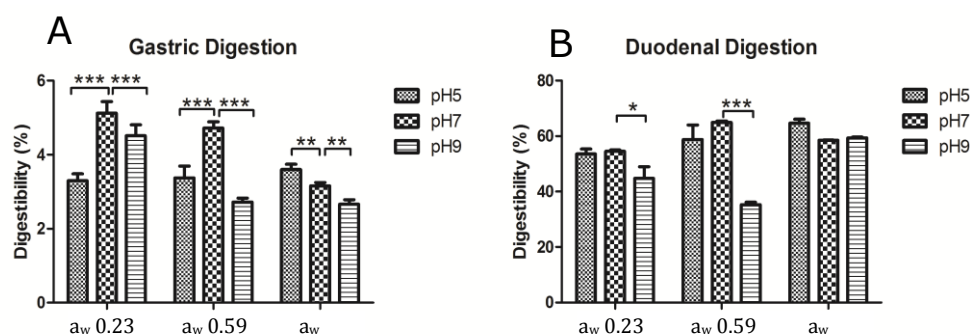


Figure 3.3. Gastric (A) and duodenal (B) digestibility of extensively heated (130°C for 10 min) whey proteins using an infant GI digestion model. The digestibility was calculated by OPA. Error bars represent standard deviations of duplicate measurements. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Heat treatment may increase exposure of peptide bonds to digestive enzymes, but the improvement in digestibility would be offset by the thermal-induced decrease in

protein solubility [39]. Besides, the formation of lactulosyllysine, the aggregation of proteins via cross-linking, and newly formed disulfide bonds, may also inhibit the action of digestive enzymes. Consequently, no significant differences in digestibility among samples heated at various pH would be expected. However, Fig. 3.4 revealed that the differences in pH of the samples led to altered peptides formed during digestion (Fig. 3.4A), whereas different water activity of the samples did not result in any differences in peptides (Fig. 3.4B). The study of Meltretter et al. indicated that both heating condition and samples composition affects the glycation sites [40]. As a result, the peptides formed during digestion may possess different biological functions. To our knowledge, this result (Fig. 3.4) is the first report describing that the pH of whey protein samples during heating may affect the peptide composition after digestion. This can be explained by the fact that the formation of fragments induced by the Maillard reaction, i.e. glyoxal dialkylimine and methylglyoxal dialkylimine, depends greatly on the pH of the reaction mixture [41]. Besides, pH affects the unfolding of whey proteins (Fig. 3.2). The local environment of lysines in differently unfolded proteins differs, which may affect the propensity for glycation. Both of these two factors may result in different glycated protein products. The glycation subsequently modifies protease action, resulting in different peptide profiles. In addition, heat treatment-induced disulphide bond interchanges [42] and aggregation between proteins is also pH dependent and could thereby affect the availability of enzymatic cleavage sites. Therefore, heat treatment on the samples prepared at pH 5 did not seem to reduce the extent of GI digestibility of whey proteins, but there were differences in cleavage patterns of peptides among the samples prepared at different pH.

It is well acknowledged that heat treatment of milk proteins induces a variety of modifications, reducing the nutritional value of dairy products. Nevertheless, heat treatments may not only affect IgE triggering potential of allergens and resulting peptides, but could also influence the consecutive immune responses. In this respect, the bioactive peptides which possess immune-modulatory activities are of special interests [43]. Therefore, differences in the peptide composition as found in this study should be further studied to investigate their sequences and bioactivities.

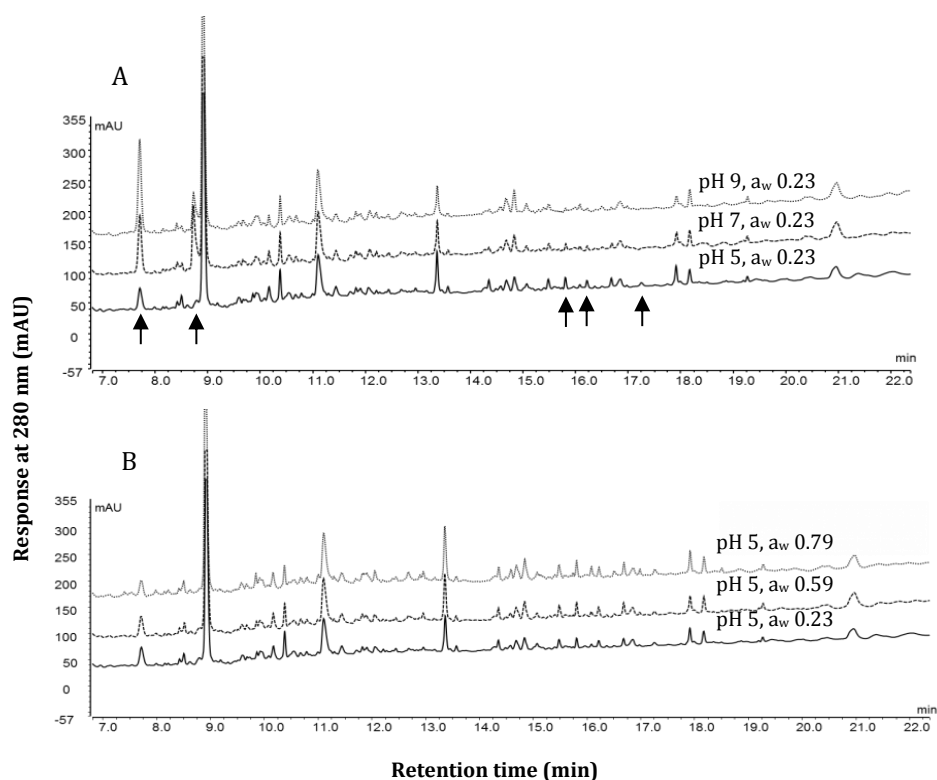


Figure 3.4. RP-HPLC chromatogram of the duodenal digests of: (A) samples with the same a_w but differing in pH and (B) samples with the same pH but differing in water activity. The arrows indicate the main differences in the peptide patterns.

Formation of sRAGE-binding ligands

Influence of pH on the formation of sRAGE-binding ligands

As shown in Fig. 3.5 (5A-5C), the formation of sRAGE-binding ligands was limited in the samples prepared at pH 5 and different a_w . Higher water activity resulted in reduced formation of sRAGE-binding ligands, which is in line with our previous study [21]. The amount of sRAGE-binding ligands in different a_w groups correlates to the amount of large aggregates as shown in Fig. 3.2B, where less aggregates cannot enter the gel were detected in the samples with a_w 0.79. This correlation indicates that the formation of aggregates via glycation-induced covalent crosslinking rather than via

thiol groups accounts for the differences in the amount of large aggregates in samples with different a_w . This is in agreement with our previous report that there is no significant difference in the amount of accessible SH between groups with different a_w after 10 min of heating [21]. The sRAGE-binding capacity of the glycated samples which have higher digestibility decreased after GI digestion (Fig. 3.5D). The samples with relatively low GI digestibility (Fig. 3.3B), however, showed increased sRAGE-binding capacities after GI digestion (Fig. 3.5D). Since glycation makes digestion more difficult, smaller digested fragments are less likely to be glycated. Glycated proteins were partly degraded to fragments and sRAGE-binding sites originally hidden inside the molecule may subsequently become more accessible to RAGE after protease hydrolysis, possibly explaining the slight increase in the sRAGE-binding capacity of the samples (Fig. 3.5D).

The reactive dicarbonyls (mainly α -dicarbonyl) are the central intermediates that accelerate AGE formation [16]. In a protein/reducing sugar system, these dicarbonyls may be formed via two pathways: from Amadori products or from Schiff bases via the Namiki pathway [44] or only from reducing sugars via caramelization [45]. The quantitative contribution of individual pathways on the formation of reactive dicarbonyls is still under debate. However, the generation of dicarbonyls from lactulose during heating of milk was reported as the more important pathway [46]. According to the report of Hodge, furfurals and related derivatives are preferentially generated under acidic conditions, while reductones and reactive dicarbonyls are mainly generated via the Namiki pathway under alkaline conditions [47]. In our bovine whey protein/lactose system, formation of sRAGE-binding ligands is limited in acidic condition, especially in the samples with a_w of 0.23 (Fig. 3.5), indicating that less reactive dicarbonyls were formed in the samples heated at pH 5. These results may be explained by the pH dependence of the formation of reactive dicarbonyls via the Namiki pathway, under the heating conditions applied in this study. From this pathway, the reactive dicarbonyls and the subsequent AGEs were generated to a larger extent.

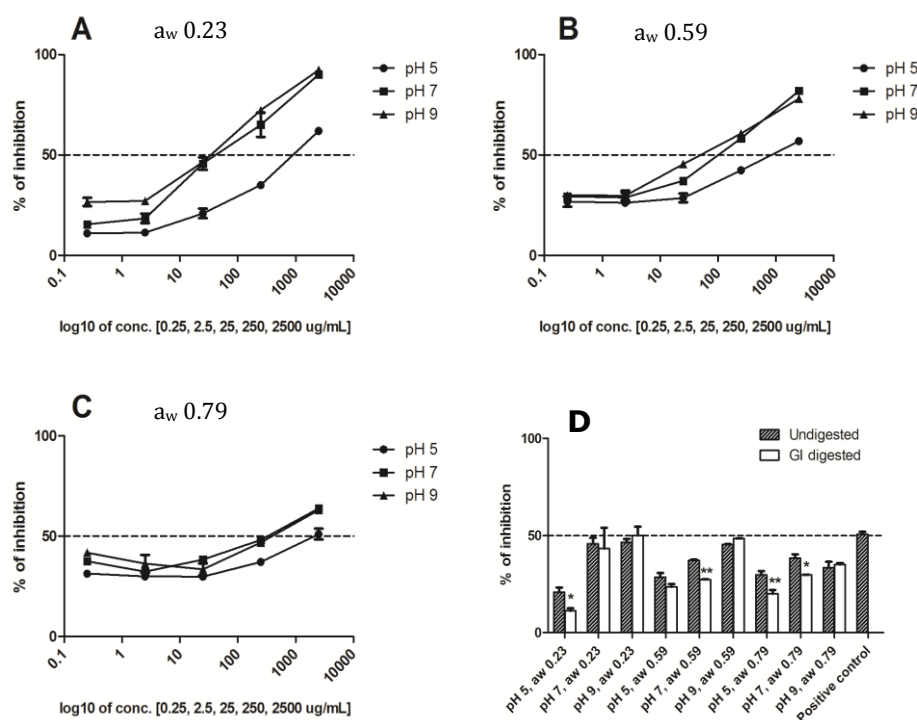


Figure 3.5. Formation of sRAGE-binding ligands at a_w 0.23 (A), 0.59 (B) and 0.79 (C). The inhibitory abilities of the heated samples before and after gastrointestinal digestion (D). Error bars represent standard deviations of triplicate measurements. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$. The value of negative sample ovalbumin (OVA) has been subtracted. The positive control sample was prepared by heating WPI and lactose with a_w 0.23 at pH 9 and 130°C for 15 min, which showed highest sRAGE-binding capacity in our trial experiment.

Influence of aggregation on the amount of sRAGE-binding ligands

SEC profiles on a Superdex 75 column are shown in Fig. 3.6. According to the standards, peak 1 and peak 2 eluted between 6 and 8 mL represent large aggregates, followed by peak 3 and peak 4 which stand for BSA and polymers of BLG and α -lactalbumin. Peak 5 represents monomeric BLG. More large aggregates were observed in the samples prepared at pH 7 and pH 9. The average size of the aggregates was previously also shown to increase with pH during dry heating [22]. Mine explained

that protein had a higher propensity for polymerisation via sulphhydryl/disulphide interchange reactions at higher pH (pH 9.5), resulting in an increased rate of denaturation/aggregation of the protein and consequently resulting in the formation of larger aggregates [48]. In the samples prepared at pH 7 and pH 9, more sRAGE-binding ligands were observed (Fig. 3.5), indicating that the formation of sRAGE-binding ligands dependent on thermal-induced aggregation. RAGE is considered to be a pattern recognition receptor, and the similarities to members of the family of Toll-like receptors has previously been considered [49].

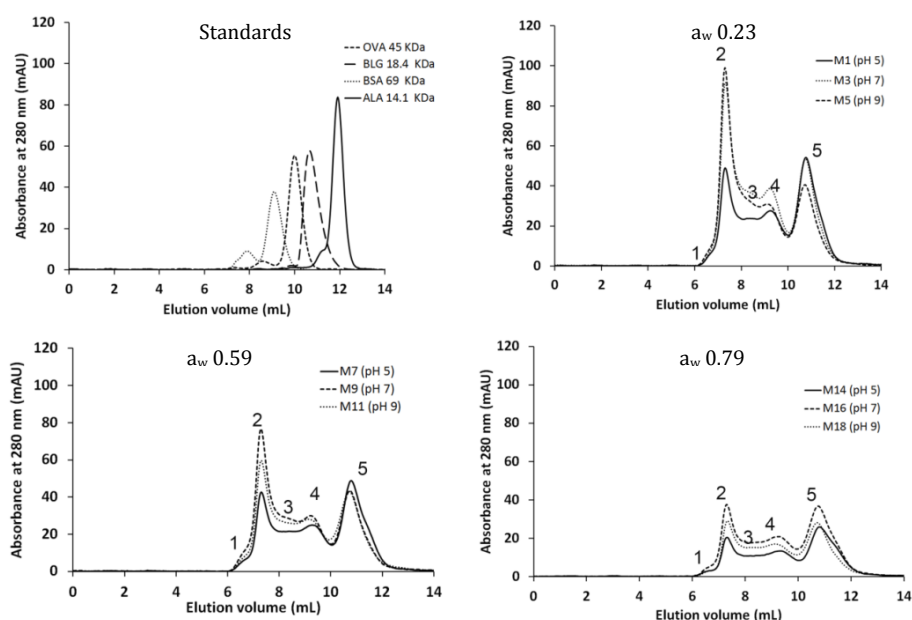


Figure 3.6. Size exclusion chromatography profiles (Superdex 75 column) of glycosylated whey protein samples (M) with various a_w : 0.23 (M1 with pH 5, M3 with pH 7, M5 with pH 9), 0.59 (M7 with pH 5, M9 with pH 7, M11 with pH 9) and 0.79 (M14 with pH 5, M16 with pH 7, M18 with pH 9).

Conformational structures, such as β -sheets and fibrils, rather the primary sequence of proteins, are recognized by RAGE [50], suggesting the influence of changes in the content of β -sheets on the RAGE-binding capacity. Since β -sheets induce a better environment for intermolecular interactions via hydrogen bonding [51], a thermal-

induced increase in the content of β -sheets also favours the aggregation of proteins. The negative peak in the region of 210-220 nm is characteristic of proteins rich in β -sheet [52]. As shown in Fig. 3.7, the content of β -sheets slightly increased with prolonged heating. This may explain the increase in the sRAGE-binding capacity and aggregation of dry heated whey proteins/lactose.

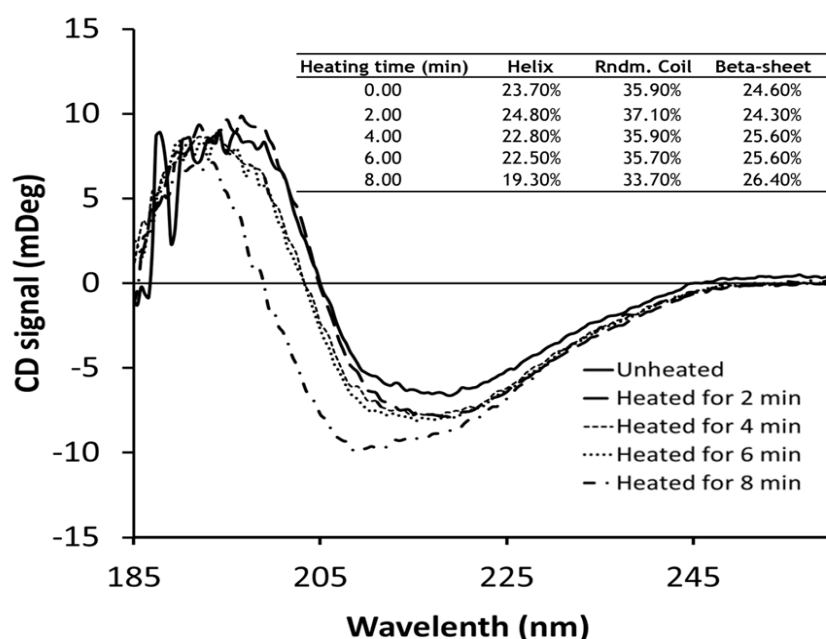


Figure 3.7. Far-UV circular dichroism spectra of BLG and lactose mixture heated for 0, 2, 4, 6 and 8 min. The secondary structures content of heated samples was calculated by CDNN software and shown in the top of the figure.

In the study of Morgan et al., 8-anilino-1-naphthalenesulfonic acid (ANS) binding assay and proteolytic susceptibility analysis were performed to study the influence of heat treatment and glycation at low temperature (50°C) on the structure of BLG in dry state. They reported that heat treatment slightly affected the structures and functionalities of BLG, but did not affect glycation [53]. Glycation induces chemical modifications on lysine side chains without altering the conformational structure of proteins, but increasing the hydrophobicity and changes the protein charge [47].

Consequently, native-like aggregation, which consists of elements with preserved global fold and flexibility, native-like global shape, and size-related parameters, will be formed and contributes to the formation of insoluble aggregates. This native-like aggregation process was also reported in several previous studies [54, 55], but the temperature applied in these studies was relatively low (37°C) and the incubation time was much longer than in our study. Since the AGE/RAGE interaction, in which RAGE recognizes conformational structures rather than amino acid sequences, does not seem to be influenced by protein aggregation in this study, it could be speculated that the glycation-induced aggregation processes affect the conformational structure of proteins to a limited extent.

It is noteworthy that the PBS soluble fraction of proteins underwent an unfolding process during dry heat treatment, resulting in the increased accessible amino acids with the increase of pH (Fig. 3.2). However, more accessible amino acids were observed in the samples heated at pH 9 (Fig. 3.2A), which seems in conflict with the SEC results (Fig. 3.6) that showed more aggregates in these samples. The unfolding process is transient and may therefore not be required for the subsequent aggregation [54], suggesting that the thermal-induced unfolding process alone will not affect the formation of native-like aggregates and sRAGE-binding capacity.

In conclusion, heat treatment on the samples prepared at pH 5 did not reduce the extent of GI digestibility of whey proteins. However, the peptide composition of the digests was different among the samples prepared at different pH. To our knowledge, this result is the first report describing that the pH of the whey protein/lactose mixtures during dry heating affects the peptide composition after digestion. Whey proteins possess enhanced thermodynamic stability at pH 5. The formation of sRAGE-binding ligands in this whey protein/lactose model may be related to the content of β -sheets, which is limited when heating in acidic conditions. The sRAGE-binding capacity of sRAGE-binding ligands generated in extensive heating was affected by simulated in vitro infant GI digestion and correlates with the digestibility of samples, and it was dependent on thermal-induced aggregation. In light of these findings, it is suggested that a high moisture and low pH environment may be effective strategies to limit the formation of sRAGE-binding ligands and retain digestibility of whey proteins after extensive heating.

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Chapter 4

Controlled Maillard Reaction upon Extensive Dry Heating
Potentially Confers Anti-inflammatory Properties on β -
lactoglobulin as Tested with THP-1 Macrophages



Abstract

Ingestion of extensively heated milk was reported to be related to an increased tolerance to regular milk in cow's milk allergic children. This study aimed to investigate the immunogenicity of β -lactoglobulin (BLG), which is considered as one of the most important milk allergens, under controlled extensive heating. Glycated BLG was prepared by heating with lactose at 130°C at a water activity (a_w) of 0.23 and 0.59, respectively. The formation of advanced glycation end products (AGEs) was tested by an sRAGE-binding assay. IgE-binding capacity of native and glycated BLG was measured by dot-blot. Polarization of THP-1 monocytes and gene expression of M1 and M2 macrophages upon stimulation with different forms of BLG were tested to evaluate the immunogenicity of BLG. Results showed that more AGEs were formed in the extensively dry heated samples at a_w 0.23. Glycated samples showed an enhanced IgE-binding capacity, which correlated to the content of sRAGE-binding ligands. Glycation of BLG was found to reduce the expression of pro-inflammatory TNF- α , and increase the expression of anti-inflammatory TGF- β in M1 and M2 macrophages. These results indicate that glycation of BLG under controlled temperature and water activity conditions may retard polarisation of macrophages towards the pro-inflammatory M1-phenotype.

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Introduction

Food allergy is a significant public health concern. It affects more than 1-2% but less than 10% of the population [1], and milk allergies are among the most common. About 75% of the infants who have IgE-mediated cow's milk allergy (CMA) in their first year of life outgrow their allergies by the second decade [2]. However, an increasing failure of children to outgrow their CMA was observed in a referral practice [3], which may explain the increase in CMA prevalence reported in several studies [4, 5].

Ingestion of baked milk was related to an increased development of tolerance to regular milk by ages of 5 years [6]. In addition, long-term follow-up showed that the development of immune tolerance to milk allergens could be accelerated by regular ingestion of the baked form of cow's milk allergens [7]. Decreased SPT wheal size and specific IgE levels, and increased specific IgG4 levels were observed in subjects who regularly ingest baked milk, similar to the changes seen in oral immunotherapy (OIT) trials [8]. These studies suggested the potential of baked milk as a form of OIT, accelerating the process to outgrow milk. A comparable observation was done for baked egg [9]. Compared to strict avoidance of allergens and OIT in the traditional sense with gradually increasing amounts of unmodified allergens, adding the extensively heated (baked) form of milk and egg allergens into the diets of allergic subjects can support the development of a form of tolerance and improve their quality of life.

Most children who are allergic to regular milk or egg can tolerate the baked form of the allergen [7, 10]. The baking process of a food such as biscuit, starts from a homogeneous dough with higher a_w , undergoes nonuniform water loss and a varying temperature distribution, and ends up with a product with extensively heated surface and a moderately heated inner part. This process may decrease IgE or IgG-binding capacity of allergens by destroying the conformational epitopes. The study of Bartnikas et al. reported mild symptoms or no use of epinephrine when challenging with baked milk [10]. Nowak et al. reported that 8 out of 23 (35%) baked milk-reactive subjects received epinephrine during baked milk oral food challenges [8], also indicating the decreased allergenicity of baked milk and its potential use in future clinical practice. However, there is no report that describes a standard approach of preparing "baked allergens" up to date. This may lead to inconsistencies in diagnostic testing for predicting baked milk reactivity and a debate over whether baked milk can

be introduced at home or if a physician-supervised challenge should be performed first. Although this baked milk may be involved in (acceleration of) the development of tolerance, the tested products are complex and composed of multiple ingredients that have been subjected to a heat treatment (baking). A possible mechanism for and identification of components that may be involved in the accelerated tolerance induction remain obscure.

In the evaluation of allergenicity of baked allergens, their IgG and/or IgE-binding capacity are normally tested. However, IgE has specificity for the anionic character and glycated lysine provided by AGE adducts, which may contribute to increased IgE-binding of heated allergens, as was observed in some studies [11, 12]. The IgE-binding capacity does not reflect the propensity of extensively heated allergens to initiate an immune reaction, and the question still remains as to whether AGE adducts are immunogenic. Alternatively, functional tests on immune cells, such as basophils, macrophages, dendritic cells, and mast cells, were adopted to evaluate the immunological properties of AGEs [13-15], but the results were inconclusive or even in contradiction with each other [16, 17]. The study of Chun et al. suggested that the glycated whey proteins provide immunomodulatory activities [17], whereas an enhanced production of pro-inflammatory cytokines by macrophages was reported in the study of Pertyńska-Marczewska et al. [16]. Macrophages are tissue-resident immune cells capable of phagocytosis and antigen delivery to the immune system. Phagocytosis and receptor mediated endocytosis by macrophages are essential for the initiation of immune responses [18]. Thermal modification of allergens may alter binding to receptors on the macrophage, affecting their recognition, uptake, and processing, and thereby possibly altering activation of signalling pathways. However, little information is available regarding direct influences of AGEs on immune cells, including macrophages.

Activated macrophages may exhibit various phenotypes with different immunological functions. Classically activated macrophages (M1) and alternatively activated macrophages (M2) are two common groups of activated macrophages [19]. M1 polarized macrophages are characterized by expression of pro-inflammatory mediators, such as interleukin-6 (IL-6), interleukin-1 β (IL-1 β), and tumour necrosis factor- α (TNF- α), while M2 polarized macrophages are characterized by expression of

immunomodulatory cytokines including transforming growth factor- β (TGF- β), DC-SIGN, and interleukin-10 (IL-10) [20, 21]. Several studies have reported that the phenotype of macrophages is plastic [22]. IL-10/TGF- β -modified macrophages produce anti-inflammatory cytokines, and induce the development of regulatory T (Treg) cells [23], which are considered as the primary factor of the development of oral immune tolerance. Tissue macrophages co-expressing TGF- β are capable to sample antigens and present them to CD4⁺ T cells, resulting in the generation of Foxp3⁺ Treg cells, and subsequently contribute to the induction of immune tolerance [24]. These studies indicate the important role of macrophages and their cytokine expression in the development of immune tolerance. In this study, the effect of glycation of BLG on polarisation of macrophages was studied, to contribute to elucidating the molecular mechanism underlying accelerated resolution of immune tolerance induced by baked milk [25].

Materials and methods

Chemicals and materials

Beta-lactoglobulin (L3908, more than 90% pure) was purchased from Sigma (Zwijndrecht, The Netherlands). Lactose (10039-26-6, Lactose monohydrate) was obtained from Millipore (Amsterdam-Zuidoost, The Netherlands). NuPAGE 12% Bis-Tris Gels, MES running buffer, and sample buffer containing lithium dodecyl sulphate at a pH of 8.4 for SDS-PAGE were provided by Life Technology (Carlsbad, USA). A molecular marker set of 3.5-240 kDa was obtained from Jena Bioscience (Jena, Germany). Soluble receptor for AGEs (RD172116100, sRAGE) was purchased from Biovendor (Brno, Czech Republic). Anti-RAGE, monoclonal mouse IgG2B human RAGE antibody (MAB11451) and detection antibody were obtained from R&D systems (Minneapolis, USA). Polyclonal goat anti-mouse HRP-conjugated antibody (P0447) was obtained from DAKO (Glostrup, Denmark). Human monocytic leukaemia cell line was from American Type Culture Collection (Manassas, USA). All the other chemicals were from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Sample preparation

BLG and lactose were dissolved in PBS (pH 9) at a protein to lactose ratio of 1:1.5 (w/w), which is similar to the protein to lactose ratio in milk. BLG samples without lactose were also included. The solutions were lyophilised. Three groups of such lyophilised samples (0.25 g) were stored in desiccators containing saturated potassium acetate and sodium bromide solution for two weeks at room temperature to obtain initial a_w of 0.23 and 0.59 at 20°C, respectively. Powders in screw-cap test tube (Schott GL18) were heated at 130°C for 7 min in a heating block (Labtherm Graphit, Liebisch, Germany). The samples were prepared in duplicate. Unheated samples in each group were also prepared as controls. Then the PBS soluble protein samples were obtained and characterized by Dumas and SDS-PAGE using the methods we reported previously [26].

To eliminate the influence of lipopolysaccharide (LPS) on the subsequent cell experiments, Triton X-114 (TX-114) was used for its removal according to the method of Teodorowicz et al. (publication in preparation). TX-114 was added to the protein solution at a final TX-114 concentration of 2% (v/v), followed by an incubation at 4°C for 30 min under constant stirring. After that, the samples were incubated for 10 min in a 37°C water bath followed by centrifugation at 20000 × g for 10 min at room temperature. The upper layer was collected and the remaining LPS concentration was determined by spectrophotometer using absorption at 280 nm. To remove the TX-114 in the collected supernatant, 0.3 g Bio-Beads SM-2 (Bio-Rad, cat. # 152-8920) with high affinity for Triton were added to 5 mL samples and incubated at 4°C overnight under constant stirring.

Size exclusion chromatography (SEC)

Separation of glycosylated whey protein aggregates by size was carried out by SEC on an AKTA micro system (GE Healthcare, Uppsala, Sweden) equipped with a Superdex 75 HR 10/300 column (GE Healthcare) as described by Butré et al [27]. The experiments were performed at 20°C with 10 mM sodium phosphate buffer with 150 mM NaCl at pH 8.0 (filtered through a 0.2µm membrane), and a flow rate of 800µL/min. The elution of the injected samples (50µL) was monitored at 280 nm. Ovalbumin (45 kDa),

β -lactoglobulin (BLG, 18.4 kDa), bovine serum albumin (69 kDa), and α -lactalbumin (14.1 kDa) were used as standards to determine the content of peaks.

sRAGE-binding assay

The sRAGE-binding assay was used to measure the binding between the soluble form of RAGE receptor and the heated WPC, both before and after digestion. The specificity was tested by creating competition between a known high affinity ligand coated on the plate and the possible ligand-dry heated BLG samples. The transparent ELISA plates (Geiner Bio-One, cat. # 655061) were coated with glycosylated 90 (soy protein extract glycosylated with glucose, gSPE) in 1.5 mM of sodium carbonate buffer pH 9.6 overnight at 4°C. Next day the plate was washed with PBS containing 0.05% (v/v) Tween-20 and the plate was blocked with 3% BSA for 1 h at room temperature. BLG samples at a number of different dilutions (0.25, 2.5, 25, 250, 2500 μ g/mL) were pre-incubated (45 min, 37°C) with sRAGE (recombinantly produced in *E. coli*. Biovendor RD172116100, 1 μ g/mL) in the dilution buffer (1.5% BSA and 0.025% Tween-20 in PBS). After blocking, the coated ELISA plate was washed and then the pre-incubation mixture was added followed by 1 h of incubation at 37°C. The plate was washed and anti-RAGE antibody (monoclonal mouse IgG2B human RAGE antibody MAB11451; 1 μ g/mL) was added and incubated for 30 min on the shaker at room temperature. After washing the detection antibody (polyclonal goat anti-mouse HRP-conjugated P0447; 1:1000) was added and incubated for 30 min on the shaker at room temperature. The plate was washed and TMB substrate (3,3',5,5'-tetramethylbenzidine) was added and incubated for 15 min. The colouring was stopped using 2% HCl. The plate was read using the Filtermax F5 at 450 nm with 620 nm as reference. The percentage of inhibition was calculated using the formula: $((OD_{sRAGE} - OD_{sample}) / OD_{sRAGE}) * 100$, where OD sRAGE was the signal obtained for sRAGE incubated without the competition agent (BLG sample) in the dilution buffer (maximal signal). All tests were performed two times in triplicate.

Immunoassay by dot-blot

Eleven sera obtained from milk allergic patients were tested for specificity to BLG using a dot-blot immunoassay. Three sera from patients allergic to non-milk allergens (Skim 2012-08, Skim 2012-05, Skim 2012-12) and three non-allergic sera (JW, AT, and

RG) were used as controls. The sera with BLG-specific IgE amount higher than the control sera were pooled. Native and glycated BLG samples (0.25 µg) were spotted on the nitrocellulose membrane with 3 × 3 mm grid. A Tris-buffered saline (TBS)-only sample was included as negative control. The membrane was dried at 37°C for 1 h and unbound protein-binding sites were blocked by 3% gelatine in TBS. The dried membrane was washed five times with TBS containing 0.05% (v/v) Tween-20 (TBST) followed by one TBS wash. The pooled sera were diluted 1:10 in TBS and incubated with the membrane overnight at room temperature. After washing, the membrane was incubated with 1:15000 diluted rabbit anti-human IgE antibody for 1 h at room temperature. AP-conjugated polyclonal goat anti-rabbit antibody was 1:20000 diluted in TBS and incubated with the membrane. After another washing step with TBS, the spots were visualised by incubating the membrane with SigmaFast™ BCIP/NBT for 10 min at room temperature. Then the membrane was washed with MilliQ water and dried for 1 h at 37°C. Colour intensity of the spots was determined using BIO-RAD Universal Hood II Gel Imager and analysed with Imagelab 4.1 software.

THP-1 cell culture

Human monocytic leukaemia THP-1 cells were seeded in 75-cm² flasks at a density of $1.5 \times 10^5/\text{cm}^2$. The medium used was RPMI 1640 culture medium (cat. # 12-702Q, Lonza, Switzerland) supplemented with 10% of fetal bovine serum (cat. # 10270-098, Life Technologies) and 1% of penicillin/streptomycin (cat. # 15140-122, Life Technologies). Cells were cultured at 37°C in 5% CO₂ in a humidified incubator. The medium was replaced twice a week.

THP-1 cell differentiation

The THP-1 monocytes were transferred to a 24 well plate (Greiner bio-one) at 5×10^5 cells/well and treated with 100 ng/mL phorbol 12-myristate 13-acetate for 48 h. Differentiated macrophages were washed and subsequently rested for 24 h. Macrophage polarization was obtained by replacing the culture medium with RPMI 1640 medium supplemented with 20 ng/mL IFN-γ and 1 µg/mL LPS (for M1 polarization) or 20 ng/mL IL-4 (for M2 polarization) and culturing the resting macrophages (M0) for an additional 18 h. The harvested macrophages (M0, M1, and

M2) were incubated with fresh RPMI 1640 medium containing the BLG samples (LPS-free) at a concentration of 100µg/mL for 24 h. After that, the cells were sedimented by centrifugation and the cell pellet was collected for further experiments.

Quantitative real time PCR (qPCR) analysis

Total cellular RNA was extracted from the THP-1 macrophages using RNeasy Mini kit (Qiagen, USA) with an RNase-free DNase (Qiagen, cat. number 79254) treatment according to the manufacture's instruction. Concentration of the extracted RNA was measured by agarose gel electrophoresis and NanoDrop. The total RNA was reverse transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad iScript, cat. number 170-8891). Afterwards, a mixture of 200 ng of the synthesized cDNA, 10µL primer pairs, and 20µL IQTM SYBR Green Supermix (Bio-Rad) was pre-heated for 90 s at 90°C, followed by PCR for 40 cycles. Amplified PCR products were separated on a 1% agarose gel and visualized by GelStar staining (BMA Products).

Statistical analysis

Two independent experiments for two batches of heat-treated BLG and BLG/lactose samples were performed in duplicate or triplicate. Data were expressed as mean ± SD. Two-way ANOVA was used to determine the statistical significance of the differences. Significance was defined at $p < 0.05$. Statistical analysis was performed by GraphPad Prism 4 software.

Results and discussion

Characteristics of lactose glycated BLG.

Extensive heat treatment leads to the formation of aggregates from BLG in the presence or absence of lactose, with most of the BLG present in a monomeric form in the unheated samples and the samples heated without lactose (Fig. 4.1). Protein dimers and polymers can be clearly observed in the samples of BLG heated without lactose, with the amount of monomeric BLG decreasing accordingly. All the samples were stored for 14 days at room temperature to reach an a_w of 0.23 and 0.59, respectively. This long-term storage caused no changes in the lactose-free BLG and the BLG with lactose at a_w 0.23. However, the apparent molecular weight of the BLG

increased in the samples at a_w 0.59 (Fig. 4.1B), indicating that the reaction between BLG and lactose already started during the 14-day storage at room temperature of these samples. The reaction between sugars and protein is affected, directly or indirectly, by the water activity in a food. The concentration of reactants, the mobility of reactants, and heating conditions (temperature, pressure, pH, etc.) affect a reaction rate. At higher a_w , the mobility and reactivity of water are relatively high if it acts as a reactant, and the reaction volume is less limited if water acts as a solvent. It is difficult to predict which effect dominates in determining the increased reaction rate in the samples with a_w 0.59 during the 14-day storage at room temperature, as seen in Fig. 4.1B.

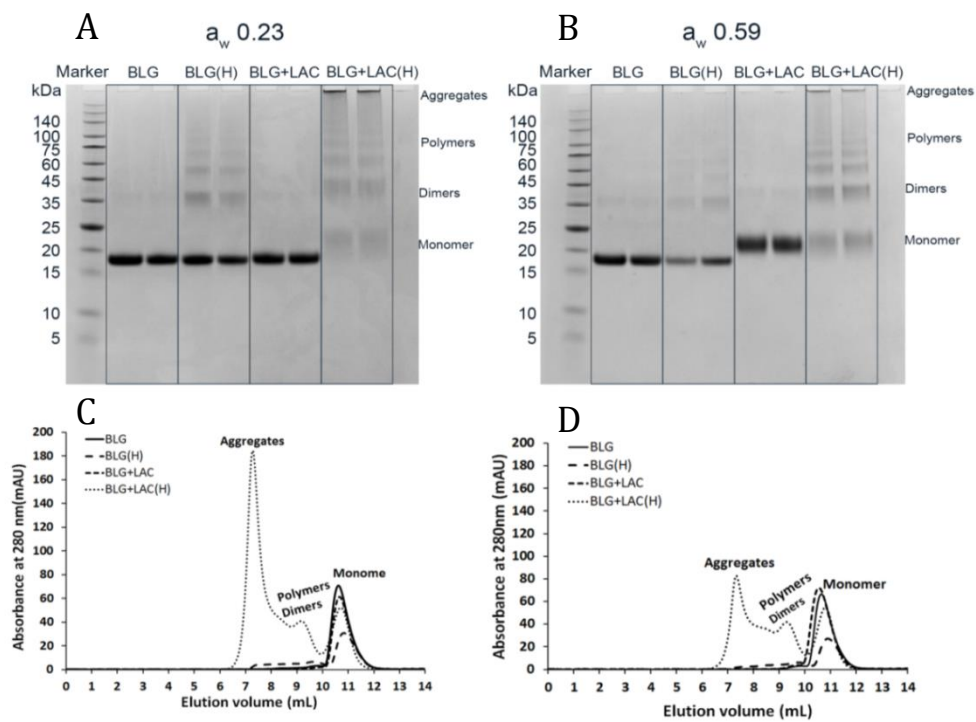


Figure 4.1. Formation of aggregates upon extensive heat treatment of BLG with or without lactose (LAC) as determined by SDS-PAGE (A, B) and size-exclusion chromatography (SEC) (C, D).

In the heated BLG with lactose, monomeric BLG dramatically decreased at both a_w 0.23 and 0.59. Dry heating of these samples induced substantial aggregation of the protein. The mobility of the polymerized proteins in SDS-PAGE electrophoresis was decreased by glycation. The intensities of the bands of large aggregates were higher in the samples at a_w 0.23 than in the samples at a_w 0.59, indicating that more large aggregates were formed in the samples with a_w 0.23. To confirm the results obtained by SDS-PAGE, SEC was carried out to determine the size distribution of protein aggregates in the samples. As shown in Fig. 4.1C and Fig. 4.1D, dimers, polymers, and large aggregates were formed in the heated samples in presence of lactose. In line with the results of SDS-PAGE, more large aggregates were formed in the samples with a_w 0.23. This observation indicates that the a_w effect on the degree of Maillard reaction depended on temperature. During heat treatment, the formation of aggregates was accompanied by the degradation of Maillard reaction products. In a relatively high a_w system, free amino acids and low molecular weight peptides may have formed as a result of non-enzymatic cleavage of peptide linkage [28]. Consequently, less formation of large aggregates is to be expected in the samples with higher a_w . This may explain the observation that large aggregates were more formed in a_w 0.23 samples than in samples at a_w 0.59.

Formation of sRAGE-binding ligands

The optimum a_w for Maillard reaction is food material and temperature-dependent. In milk powder, the Maillard reaction at 40°C is maximal at a_w 0.6-0.7, whereas in whey powders, the maximum is at an a_w of 0.44 [29]. In previous studies on the influences of a_w on the Maillard reaction rate, colour and furosine were commonly used as indicators. As the interactions between RAGE and AGEs may increase RAGE expression in macrophages and elicit immune responses via a NF- κ B mediated pathway [14], the RAGE-binding capacity may be an important indicator of the ability of glycation to affect the immune responses of macrophages. Therefore, RAGE-binding ligands were measured by an sRAGE-binding assay in this study. As shown in Fig. 4.2, the inhibition of the reaction between AGE and RAGE by glycated samples increased from $48.3 \pm 3.6\%$ to $85.0 \pm 5.4\%$ in the samples at a_w 0.23, and from $37.2 \pm 9.1\%$ to 75.4% in the samples at a_w 0.59. A larger amount of sRAGE-binding ligands was observed in the samples with a_w 0.23 than in the samples with a_w 0.59. This

corresponds to the results of SDS-PAGE and SEC, which indicated that more aggregates were formed in glycated samples at a_w 0.23 than at a_w 0.59.

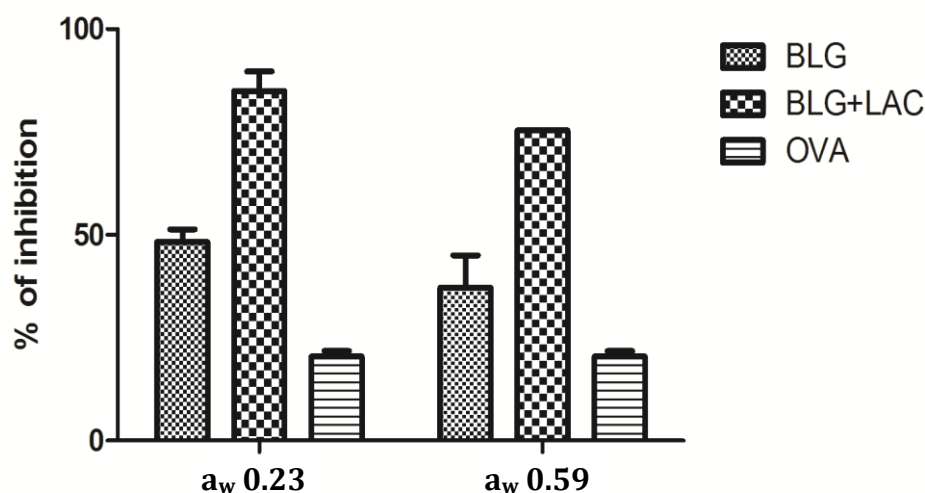


Figure 4.2. Formation of sRAGE-binding ligands of heated β -lactoglobulin in the absence (BLG) and presence (BLG+LAC) of lactose at a_w 0.23 and 0.59. Unheated ovalbumin (OVA) was used as negative control. Error bars stand for the standard derivations of the duplicated measurements.

IgE-binding capacity

In our previous study, we reported that the IgG-binding capacity of extensively heated whey protein/lactose was decreased by glycation [26]. The IgE-binding capacity of heated BLG with and without lactose was investigated in the present study. At first, BLG specific IgE sera were selected by dot-blot immunoassay. As shown in Fig. 4.3A, 8 out of 11 sera were found to be more reactive to BLG than negative controls. These sera were pooled for the subsequent IgE-binding assay. As shown in Fig. 4.3B, all the heated samples showed a higher IgE-binding capacity than their unheated counterparts. The increase in IgE-binding capacity of glycated samples was higher than that in samples heated without lactose, which correlated to the amount of sRAGE-binding ligands (Fig. 4.2). IgE-binding assays are widely used in the evaluation of the allergenicity of heated proteins [30-32]. It was reported that heat treatment may enhance IgE-binding capacity of peanut, shrimp, and shellfish allergens,

depending on allergen and heating condition [33]. Nevertheless, glycation of other allergens, i.e. cherry allergen Pru av 1, caused a decrease in the allergenicity [34]. Also, IgE-binding of some allergens may not be affected by heat treatment under certain heating conditions. For instance, Koppelman et al. reported that heated Ara h 1 exhibited IgE-binding capacity similar to those found for native Ara h 1 [35]. However, after the heat treatment that was applied in this study, the reaction between IgE and AGEs maybe because IgE, like the scavenger receptors, has a specificity for the anionic character and glycated lysine provided by AGEs [11, 12].

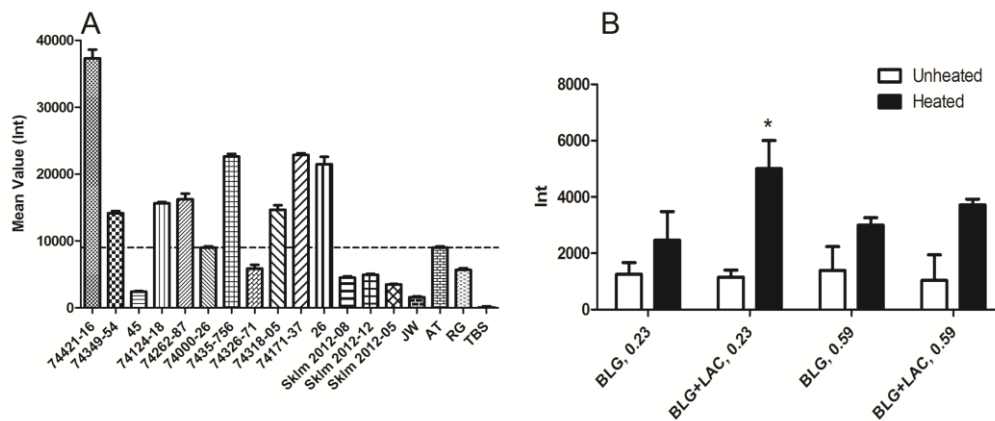


Figure 4.3. IgE-binding capacity of different forms of unheated and heated β -lactoglobulin (BLG) in the absence (BLG) and presence (BLG+LAC) of lactose (LAC) at a_w 0.23 and 0.59. Specificity of sera collected from milk-allergic patients to BLG was tested by dot-blot immunoassay. Sera with native BLG-binding capacity higher than negative controls (Sklm 2012-08, Sklm 2012-12, Sklm 2012-05, JW, AT and RG) were selected (A) and pooled for the BLG IgE-binding capacity test (B). Error bars represent standard deviations of duplicate measurements. * indicates a significant difference at $p < 0.05$.

A question remaining is whether RAGE-binding ligands are indeed allergenic. There are distinct differences between the mechanisms of IgE-AGE and IgE-antigen

interactions. Buried surface area in combination with van der Waal forces, high affinity and specificity, hydrogen-bonds and/or salt bridges contribute to the IgE-epitope interactions [36]. Upon the formation of the IgE-antigen-Fc ϵ R1 complex by these interactions, immunoreceptor tyrosine activation motifs (ITAMs) initiated degranulation and mediator release by mast cells was started. These responses may cause smooth muscle dilation, capillary disruption and other allergic symptoms [37]. Therefore, the increase in the IgE-binding capacity of AGEs does not necessarily indicate increased allergenicity. Functional assays, e.g. histamine release assay, should be used to evaluate the allergenicity of glycosylated BLG samples in general and AGEs in particular. In this study, the response of THP-1 macrophages towards RAGE-binding ligands in BLG samples was evaluated next.

Effects of glycosylated BLG on polarization of macrophages

The gene expression profile of THP-1 monocytes undergoing differentiation into mature macrophages in the presence of glycosylated BLG and the subsequent polarized activation into M1 or M2 cells were investigated. Gene expression of IL-1 β and TNF- α were considered as the hallmarks of M1 polarization, and DC-SIGN and TGF- β expression were the hallmarks of M2 polarization. As shown in Fig. 4.4, incubation of M0 cells with BLG and glycosylated BLG (at a_w 0.23 and 0.59) showed significant upregulation of the IL-1 β expression and slight upregulation of TNF- α , indicating that BLG and glycosylated BLG are effective in upregulating M1-specific genes. However, M0 macrophages stimulated with glycosylated samples expressed lower levels of IL-1 β and TNF- α gene than native and non-glycosylated BLG stimulated macrophages. It seems that native BLG and non-glycosylated BLG caused a stronger stimulation of M0 macrophages to the M1 phenotype than glycosylated BLG. The highest M2 marker gene expression (DC-SIGN and TGF- β) was observed in the macrophages stimulated by heated non-glycosylated BLG samples (Fig. 4.4C and Fig. 4.4D). Glycosylated samples stimulated M0 macrophages to express lower levels of both M1 and M2 marker genes. In summary, the BLG and heated non-glycosylated BLG are more likely to stimulate the differentiation of M0 macrophages to M1 macrophages, while glycosylated BLG down-regulated both the M1 and M2 gene expression of M0 macrophages.

It was reported that AGE-induced M1 polarization by interacting with RAGE leads to the activation of NF- κ B, which is a pleiotropic transcription factor. It can activate the transcription of C-reactive protein and maltose-binding protein, which play important roles in M1 macrophage polarization [38]. Apart from RAGE, however, five more receptors that recognize and bind AGEs have been identified [39]. Hilmenyuk et al. reported, for primary monocyte-derived DCs, that blockage of RAGE did not affect internalization of AGE-ovalbumin (AGE-OVA), while the uptake of unheated OVA and AGE-OVA was retarded by addition of inhibitors of mannose receptor, scavenger receptor, and pinocytosis [40]. The interaction of AGE and these receptors may induce cytokine production and subsequently affect macrophage polarization. Contrarily, RAGE-mediated endocytosis followed by lysosomal destruction is a very slow process [40]. This may explain the observation that lower expression of M1 marker gene was induced by glycated BLG than by unheated BLG (Fig. 4.4A and Fig. 4.4B). However, the role of the interaction between AGEs formed during glycation of BLG and other AGE receptors in the cytokine production and the subsequent polarization of macrophages needs to be further clarified.

Effects of glycated BLG on cytokine expression of M1 and M2 macrophages

The effects of glycated BLG on the gene expression of macrophages were compared with the effects of unheated BLG samples without lactose. As shown in Fig. 4.5, BLG glycated at a_w 0.59 significantly upregulated transcription of TGF- β in both M1 and M2 macrophages. Both the expression of pro-inflammatory and anti-inflammatory cytokines were determined. TGF- β is a well-acknowledged anti-inflammatory cytokine that plays an important role in the induction of Treg, while TNF- α is one of the major systemic inflammatory cytokines secreted by macrophages upon acute phase stimulation of pro-inflammatory molecules [41]. Compared to unheated lactose-free BLG samples, BLG glycated at a_w 0.23 was found to upregulate expression of M1 TGF- β and M2 TGF- β from 0.92 ± 0.02 to 1.29 ± 0.04 and 0.65 ± 0.09 to 0.85 ± 0.07 , respectively. Glycated BLG prepared at a_w 0.23 showed a weak suppressive effect on the TNF- α gene expression of M2 macrophages. Glycated BLG prepared at a_w 0.59, on the other hand, significantly suppressed the expression of the pro-inflammatory cytokine TNF- α . These results indicate that the Maillard reaction effectively decreased the pro-inflammatory response of macrophages to BLG.

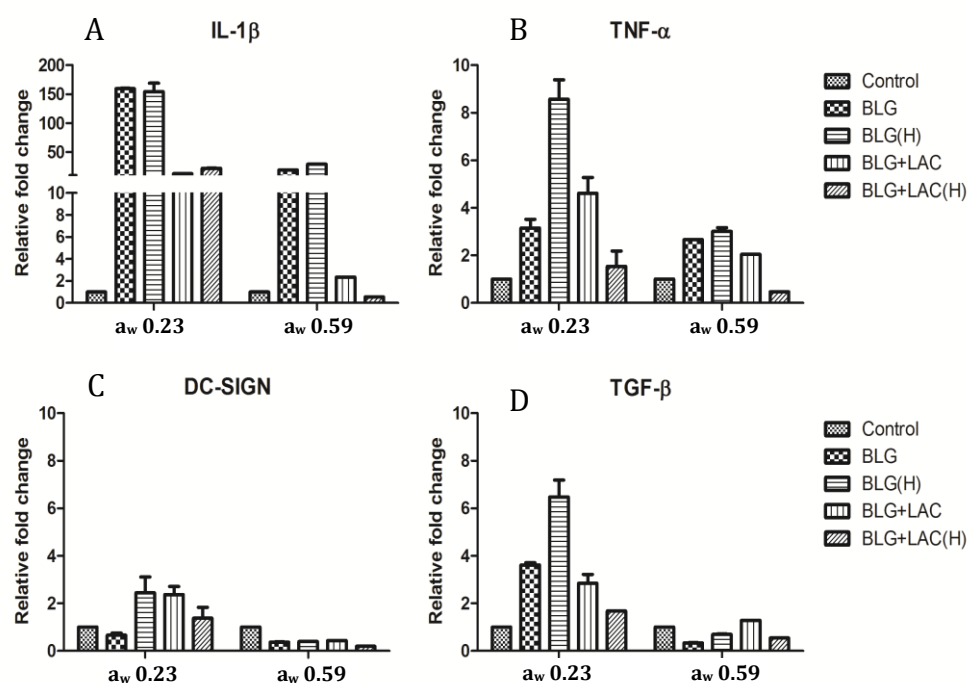


Figure 4.4. Effects of native BLG (BLG), heated BLG (BLG (H)), non-glycated BLG with lactose (BLG+LAC), and BLG glycated with lactose (BLG+LAC(H)) on M1 (A, B) and M2 (C, D) marker gene expression on THP-1 monocytes. Gene expression was expressed relative to actin- β and PUM-1 expression on M0 monocytes incubated with cell medium for 24 h.

Earlier studies reported that AGEs potentially enhance the inflammatory response of macrophages [16, 42, 43]. These authors prepared AGEs by incubating protein allergens with reducing sugars at 37°C in solution for several (≥ 2) weeks. In this study, dry heating at 130°C was applied on the mixture of BLG and lactose, and an anti-inflammatory effect of the formed reaction products on macrophage was observed. Miyazaki et al. compared the formation of scavenger receptor-binding ligands in “extensive” heating conditions (37°C for 40 weeks) and in much milder heating conditions, and found that different heating conditions resulted in the formation of different ligands of AGE receptors [39]. The interactions of different AGE-

ligands and AGE receptors may consequently cause differential responses of macrophages, and these different heating conditions may result in the formation of different Maillard reaction products. Some of these products, such as 5-hydroxymethyl-2-furfural and 5-hydroxymethyl-2-furoic acid, have been related to anti-inflammatory effects [44]. This may explain the discrepancy between the results of our study, which were obtained under extensive and well controlled heating conditions, and the results obtained after much milder heating. In the study of Nishizawa et al., fish myofibrillar protein was conjugated with alginate oligosaccharide via controlled Maillard reaction in a dry state. Similar to the results found in this study, the Maillard reaction products showed an effective anti-inflammatory response in lipopolysaccharide-stimulated RAW264.7 macrophages [45].

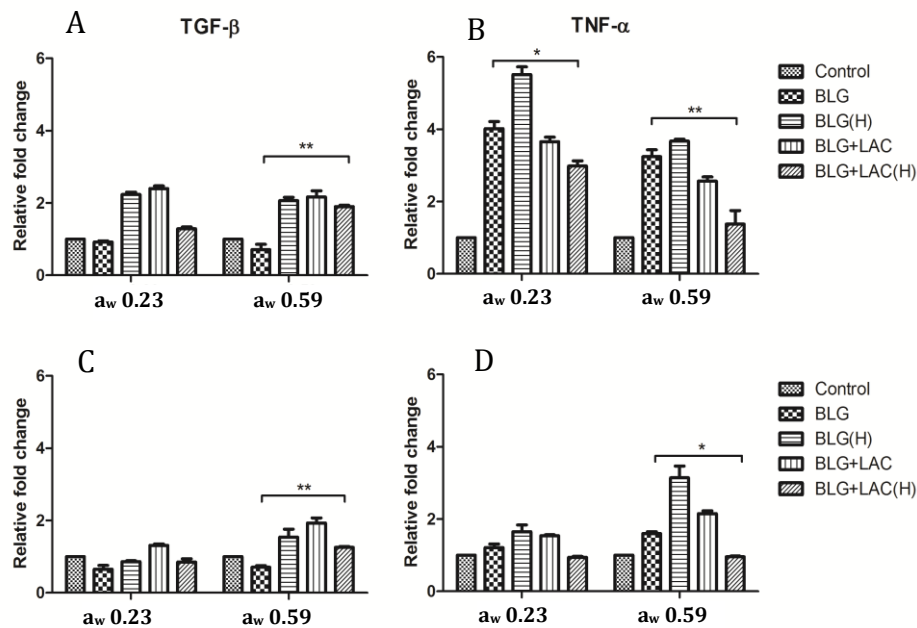


Figure 4.5. Effects of native BLG (BLG), heated BLG (BLG (H)), non-glycated BLG with lactose (BLG+LAC), and BLG glycated with lactose (BLG+LAC(H)) on anti-inflammatory (TGF-β) and pro-inflammatory (TNF-α) gene expression on M1 (A, B) and M2 (C, D) macrophages. Gene expression was expressed relative to actin-β and PUM-1 expression on M1 and M2 monocytes incubated with cell medium for 24 h. *p < 0.05; **p < 0.01.

In addition, the oral administration of Maillard reaction products of fish myofibrillar protein reduced localized acute inflammation in mice. A relatively high temperature (70°C) was applied to obtain the Maillard reaction products from the mixture of fish myofibrillar protein and alginate oligosaccharide. Under lower temperature heating conditions, however, reduced anti-inflammatory effects were observed. These results and the similar results obtained in our study indicate that extensive dry heating under controlled temperature and humidity conditions is potentially a useful method to prepare allergens with anti-inflammatory properties.

In conclusion, AGEs are formed most in the extensively dry heated samples at a_w 0.23. Glycated BLG samples showed an enhanced IgE-binding capacity, but this does not necessarily need to correlate to the immunogenicity of glycated protein allergens. The qPCR assay suggested that BLG enhances THP-1 monocytes polarization toward a pro-inflammatory M1 phenotype. Glycation of BLG under controlled temperature and water activity conditions was shown to produce components with potential anti-inflammatory properties as tested with M0, M1, and M2 macrophages.

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Chapter 5

Glycation of β -lactoglobulin reduces its allergenicity but enhances its uptake and degradation by dendritic cells



Abstract

Extensively heated milk allergens can be consumed by most allergic children and contribute to the development of immune tolerance. This study aimed to investigate the effects of glycation of β -lactoglobulin (BLG) on its allergenicity and sensitizing potential. A mixture of BLG and lactose was extensively heated under dry (water activity 0.23) and semidry (water activity 0.59) conditions. Allergenicity of glycated BLG was tested by RBL degranulation assay. Uptake and degradation of glycated BLG were tested *in vitro* in mouse bone marrow derived dendritic cells (DCs). Our results showed that glycated BLG inhibited the degranulation of basophils in a dose-dependent manner. Glycation of BLG enhanced its uptake by DCs. However, the degradation of glycated BLG was faster than unheated BLG, indicating a retarded allergen-presentation efficiency of glycated BLG by DCs. These results indicate that extensively dry heated BLG may be less allergenic, and is processed differently by DCs, than nonglycated BLG.

Manuscript in preparation: Fahui Liu, Joost J. Smit, Manon van Roest, Małgorzata Teodorowicz, Harry J. Wichers, Martinus A.J.S. van Boekel, Kasper A. Hettinga.

Introduction

Cow's milk allergy (CMA) is one of the leading causes of food allergy, especially in infants and children. The majority of CMA is mediated by allergen-specific IgE antibodies that lead to an immediate type of reaction with symptoms occurring from minutes to a few hours after consumption of allergens [1]. The current management of CMA is mainly limited to strict allergen avoidance, nutritional counselling, and managing severe allergic reactions with self-injectable epinephrine. Several allergen-specific approaches, oral immunotherapy (OIT), sublingual immunotherapy, and epicutaneous immunotherapy with food allergens, have been pursued to treat food allergies in general and CMA in particular [2].

Extensive heat treatment was reported to contribute to the resolution of CMA and has been used in clinical research [3]. Thermal treatment leads to altered structural properties of allergens, thereby affecting their allergenicity [4]. Heating induced modifications of protein allergens include partial unfolding, aggregation, cross-linking with food matrix, chemical modifications such as oxidation, and Maillard reaction [4]. These modifications are known to modulate the allergenicity of protein allergens via the destruction of epitopes and/or the formation of new epitopes (neoepitopes) [5], hence influencing IgE-binding. In the innate stage of the allergic reaction, the heat processing of allergens may contribute to the initiation of an immunological reaction as modified epitopes possibly bind to different receptors, that affect their recognition, uptake by antigen-presenting cells, thereby possibly activating different signaling pathways [6].

As the main professional antigen-presenting cell, dendritic cells (DCs) interact with allergens after they have entered the body through the gastrointestinal tract. Allergens are presented to T-cells in the context of MHC class II molecules at the cell surface, inducing T-cell immunity or tolerance [7]. In this process, uptake of allergens by DCs is essential, but little information is available regarding the mechanism(s) used by DCs to recognize and take up allergens. Allergen uptake by DCs is mediated by several mechanisms such as receptor-mediated endocytosis, macropinocytosis, and phagocytosis, depending on the nature of the allergen. C-type lectin receptors, scavenger receptors, and TLRs are considered to be involved in the allergen internalization [8]. Regarding glycated allergens, their interaction with these

receptors is not only affected by the modifications of allergen structures, but also by the carbohydrate moieties attached to the allergens. Rupa et al. reported that this carbohydrate moiety had an inhibitory effect on allergen binding *in vitro* and was associated with the suppression of the allergic response *in vivo* [9, 10]. In addition, we reported that extensive heat treatment leads to the formation of aggregates, and changes in the particle size and solubility of protein allergens [11]. These characteristics of allergens play an important role in the interactions between allergens and DCs [12]. Therefore, it can be hypothesized that glycosylated allergens could be internalized with different efficiency by DCs compared to non-glycosylated allergens.

In an attempt to have a better understanding of the role of extensively heated allergen in the induction of sensitization and in allergenicity, bovine BLG was glycosylated with lactose under intensive dry heating conditions. The allergenicity of native and glycosylated BLG was tested by degranulation assay in RBL cell line. The uptake and degradation of allergens by DCs was measured *in vitro*.

Materials and methods

Sample preparation

BLG (Sigma, Zwijndrecht, The Netherlands) and lactose (Millipore, Amsterdam-Zuidoost, The Netherlands) were dissolved in PBS pH 9 at a protein to lactose ratio of 1:1.5 (w/w), which is similar to the protein to lactose ratio in milk. BLG samples without lactose were included. The solutions were lyophilised. These lyophilised samples (0.25 g) were duplicated and stored in desiccators with saturated potassium acetate and sodium bromide solution for two weeks at room temperature to obtain a water activity (a_w) of 0.23 and 0.59, respectively. Powders in screw-cap test tube (Schott GL18) were heated at 130°C for 10 min in a heating block (Labtherm Graphit, Liebisch, Germany). Unheated samples at each water activity were used as controls. Then the PBS soluble protein was obtained and the protein concentration was measured by Dumas method as reported previously [11].

Luciferase assay

RS-ATL8 cells, an RBL cell line, were seeded in a clear-bottom white 96-well plate (5×10^4 cells/50 μ L/well) and incubated for 3 h at 37°C in a 5% CO₂ incubator. The cells were sensitized with 1:100-diluted human serum in MEM containing 10% FCS. After overnight incubation, the cells were washed once with sterile PBS, and then stimulated with 1-1000 ng/mL BLG diluted in MEM containing 10% FCS (50 μ L/well) for 3 h at 37°C in a 5% CO₂ incubator. After that, 50 μ L of luciferase substrate solution containing cell lysis reagent (ONE-Glo, Promega, Tokyo, Japan) was added to the cells, and chemiluminescence was measured using an EnVision multilabel plate reader (Perkin Elmer). Luciferase expression levels were represented as the fold increase of light units compared with the background expression after subtraction of a blank control (without cells). Measurements were done in duplicate, and the average was used for analysis.

Intracellular uptake of BLG by DCs

Six-week-old specific pathogen free female C3H/HeOJ mice were purchased from Charles Rivers (Sulzfeld, Germany). They were provided with standard laboratory food pellets and water *ad libitum*. All experiments were approved by the Animal Care Committee of the Utrecht University.

The generation of bone marrow-derived dendritic cells (BMDCs) was performed according to the method of Lutz et al. [13]. Briefly, bone marrow cells isolated from femurs and tibia were cultured for 6 days in RPMI 1640 with glutamax (Gibco) containing 10% of FBS (Multicell, Wisent Inc.), 1% of Pen/strep, 1% of Na-pyruvate, 1% MEM non-essential amino acids, 50 μ M beta-mercapto ethanol (Gibco) and 10 ng/mL GM-CSF (R&D, Oxon, UK). Glycated and non-glycated samples were dissolved in 10 mL 1 M NaHCO₃ to a final concentration of 10 mg/mL. Fluorescein isothiocyanate (FITC) at a concentration of 10 mg/mL in DMSO was prepared and added to the BLG samples at a final concentration of 1.3 mg/mL. The mixture was stored at 4°C for 24 h under constant rotation. After that, the labelled BLG was separated from the unconjugated FITC by using a gel-filtration column (GE Healthcare). Protein concentration of the labelled and unlabelled samples was measured by BCA according to the manufacturer's instructions. Labelled BLG samples were incubated at 4°C and 37°C

with BMDCs (1×10^6 cells/mL). Aliquots were taken at incubation time points of 0, 2, 5, 10, 30, 60, and 120 min for flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). The results were expressed as mean fluorescence intensity (MFI) and percentage of FITC positive DCs.

Allergen degradation by DCs

Latex beads were activated with 8% glutaraldehyde according to the manufacturer's instructions. BLG (0.5 mg/mL) was covalently coupled with activated beads. Uncoupled sites of the beads were blocked by 0.5 M glycine. BMDCs were pulsed and chased with the BLG coupled beads at a cell to beads ration of 1:5. Cells were then disrupted in detergent-containing lysis buffer at sequential time points and centrifuged at $400 \times g$ for 5 min at 4°C. The resulting supernatant containing latex beads was collected and stained with rabbit anti-BLG polyclonal antibody and anti-rabbit Alexa 488 in 96-well V-bottom microplates. The beads were analyzed by FACS, and the results were expressed as the percentage of undegraded proteins.

Statistical analysis

Two independent experiments for two batches of treated BLG/lactose samples were performed in duplicate or triplicate. Data were expressed as mean \pm SD. One-way ANOVA was used to determine the statistical significance of the differences between samples. Significance was defined at $p < 0.05$. Statistical analyses were performed using GraphPad Prism 4 software.

Results

RBL degranulation is inhibited by high concentration of glycated BLG

To evaluate the allergenicity of BLG, IgE crosslinking induced luciferase expression was measured in RS-ATL8 cell line. The expression of the reporter gene, luciferase, reflects the IgE-crosslinking capacity of proteins, which can subsequently lead to the degranulation of RBL cells. As shown in Fig. 5.1, BLG affects degranulation of RBL cells in a dose-dependent manner. The BLG samples glycated at a_w 0.23 significantly ($p < 0.05$) reduced the degranulation of RBL cells at a protein concentration of 1000 ng/mL, whereas no significant difference can be detected between the glycated and

nonglycosylated samples at protein concentration range from 1-100 ng/mL. In the samples glycosylated at a_w 0.59, glycosylated BLG at protein concentration of 100 ng/mL exhibited a significant inhibition effect on the degranulation of RBL cells. These results indicated that a high dose of glycosylated BLG is less efficient in mediating the crosslinking of IgE.

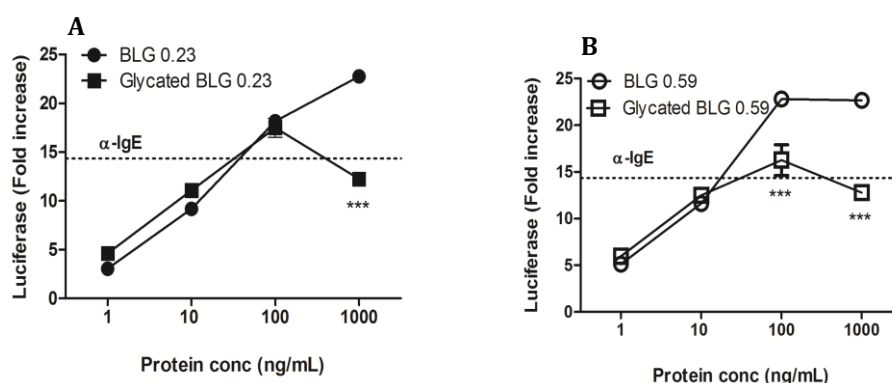


Figure 5.1. Luciferase expression of RS-ATL8 cells upon the stimulation by glycosylated and nonglycosylated BLG prepared at a_w 0.23 (A) and 0.59 (B), respectively. Data are represented as fold increase, by taking non-stimulated control as 1.0. Error bars represent SEM of three independent measurements. *** $p < 0.001$.

Uptake of BLG by BMDCs is enhanced by glycation

To evaluate the influence of glycation on the uptake efficiency of BLG by immature BMDCs, FITC-labelled BLG was incubated with BMDCs. BLG uptake was analysed by means of flow cytometry. As shown in Fig. 5.2, glycosylated BLG showed significantly enhanced uptake efficiency by BMDCs compared to nonglycosylated BLG at both 4°C and 37°C. There was no significant difference between the two groups of samples heated at a_w 0.23 and 0.59. A higher uptake efficiency of BLG at 37°C than at 4°C was observed. The uptake of glycosylated BLG increased over time at 4°C, whereas a maximum uptake was reached after 60 min when glycosylated BLG was incubated with DCs at 37°C, indicating that the uptake process was slower and prolonged at 4°C.

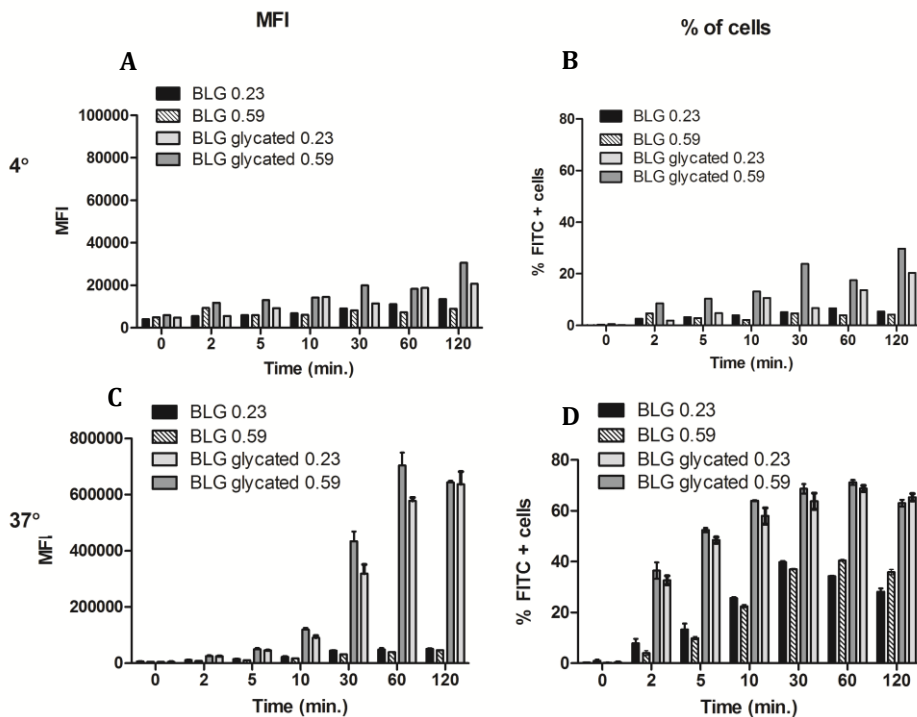


Figure 5.2. Glycated BLG uptake by BMDCs at 4°C (A, B) and 37°C (C, D) compared to the uptake of non-glycated BLG. a_w of samples before extensive heating was adjusted to 0.23 and 0.59, respectively. Results presented as mean fluorescence intensity (MFI) \pm SEM and % FITC \pm SEM labelled cells.

Degradation of BLG by immature DCs is accelerated by glycation

We analyzed the degradation of glycated and non-glycated BLG by immature BMDCs in vitro. As shown in Fig. 5.3A, glycated BLG showed accelerated degradation by BMDCs over time, independent of the a_w of the samples during heating. Internalized BLG was completely degraded after 16 hours of incubation. Cell apoptosis may affect the degradation and the subsequent allergen presentation to T cells, thus the influence of glycation on the cell viability was also evaluated in this study. As shown in Fig. 3B, there was no significant difference in cell viability observed between the groups stimulated with the glycated or nonglycated samples. These results indicated that glycation did not significantly affect cell viability at 24 h of incubation.

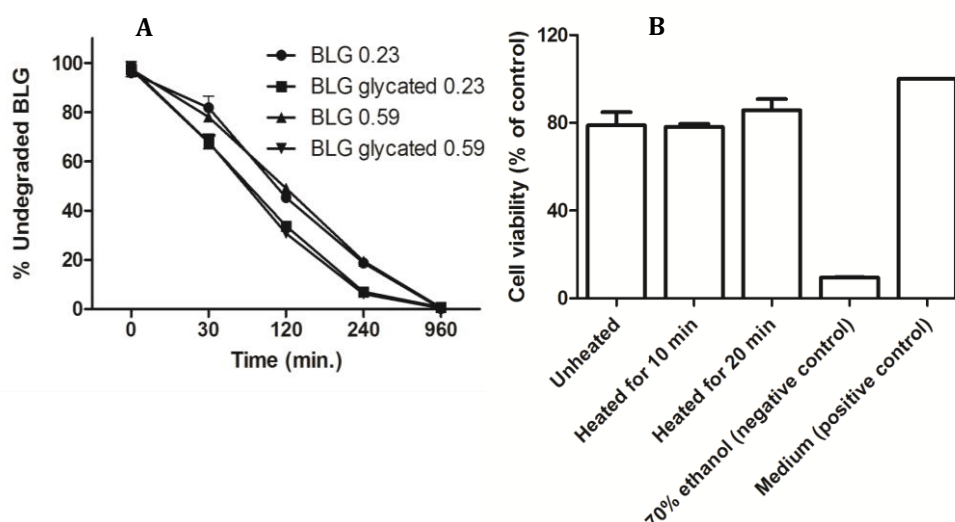


Figure 5.3. The influence of glycation on allergen degradation by immature DCs (A) and cell viability of HEK-TLR4 cells (B). The degradation result was presented as the percentage of undegradated BLG, and the cell viability was presented as the percentage of living cells in positive control. Heated and unheated samples were compared to evaluate the influence of glycation on the cell viability of HEK cells. Values are presented as mean \pm SEM.

Discussion

In chapter 4 of this thesis, an enhanced IgE-binding capacity, but reduced macrophage immunogenicity, of glycated BLG was detected. A substantial amount of advanced glycation end products (AGEs) was generated during extensive dry heating. The enhanced IgE-binding capacity, which may result from the specificity of IgE to the anionic character and glycated lysines provided by AGE adducts [15, 16], was hypothesized to be not correlated to an enhanced immunogenicity and allergenicity. The result shown in Fig. 5.1 confirmed this hypothesis, indicating that the enhanced IgE-binding capacity did not lead to an enhanced degranulation of basophils.

Several competing mechanisms determine the final allergenicity of glycated proteins. Interactions with sugars may decrease the accessibility of epitopes, whereas the thermal-induced unfolding of protein allergens or the breakdown of the Maillard reaction products may expose the conformational epitopes within the protein

molecules. In addition, glycation can potentially induce the formation of new epitopes (neoepitope) and thereby increase the allergenicity of allergens [5]. Apart from affecting immune responses of basophils through an IgE-mediated mechanism, it is noticeable that the presence of AGEs may confer additional functionality to glycated proteins and subsequently elicits immune responses via different pathways. For instance, a high concentration of glycated albumin can upregulate the expression of surface RAGE on basophils, and thereby triggers various intracellular events [17]. The interactions between AGE and RAGE can lead to the degranulation of mast cells [18], whereas there is no report on the influence of AGE/RAGE interactions on the degranulation of basophils. Our results demonstrated that a high concentration of glycated BLG enhances the IgE-crosslinking induced degranulation of basophils, but cannot exclude the potential role of AGE/RAGE interactions in this process.

In our previous study, substantial formation of AGEs was observed in the whey protein samples glycated with lactose [11]. DCs express several receptors known to bind AGEs, such as the receptor for AGEs (RAGEs), galectin-3, scavenger receptor class B type I, and CD36 [19]. Binding of AGE to RAGE is not accompanied by the internalization of its ligand, but elicits the generation of intracellular signals and subsequent immune responses [20]. Other receptors on DCs thus must be responsible for the enhanced uptake of glycated BLG, as shown in Fig. 5.2. In the study of Hilmenyuk et al., the internalization of AGE-ovalbumin was inhibited by blocking the mannose receptor, scavenger receptor, and pinocytosis, whereas the blockage of RAGE did not affect the uptake efficiency of the glycated ovalbumin [21]. Royer et al. obtained receptor-deficient DCs by gene silencing, which showed a considerable decrease in glycosylated allergen uptake. Apparently, the carbohydrate recognition domains of the mannose receptor played an essential role in this process [8]. These studies indicate that the mannose receptor may be the main receptor for the uptake of AGEs. However, the carbohydrate moieties recognition domains of the mannose receptor, which contribute to the uptake of glycated allergens, are shared by other lectin receptors [8].

In addition, allergen uptake by DCs could also proceed through macropinocytosis or phagocytosis, depending on the nature of the allergen [22]. Characteristics of allergens, such as size, surface charge, and hydrophobicity, affect the interactions between DCs and allergens and the subsequent uptake efficiency. Particles formed by aggregation

of proteins or the aggregation of proteins and reducing sugars may differ in size and surface charge depending on specific heating conditions. It was reported that particle diameters of 0.5µm and below were optimal for human DC uptake, but the uptake of larger particles could be greatly enhanced by rendering the particle surface positively charged [12]. Therefore, many factors may be responsible for the enhanced uptake of glycosylated BLG by BMDCs, but which factor dominates in this process can't be concluded from this study and needs to be further evaluated.

Compared to nonglycosylated BLG, the degradation of glycosylated BLG by DCs was significantly enhanced, as shown in Fig. 5.3A. As the initial amount of glycosylated BLG in DCs was higher than unheated BLG, both the maturation and functionalities of immature DCs may be affected. The allergen dose and the maturation state of DCs could subsequently be involved in determining whether a Th1 or Th2 response develops [23], which may influence the development of the immune tolerance. In addition, the delayed degradation of allergens enhances the allergen presentation to T cells [24], thus the enhanced degradation of glycosylated BLG may lead to a retarded allergen-presentation efficiency.

Both the glycosylated and nonglycosylated BLG were completely degraded by DCs after 16 h of incubation (Fig. 5.3A). It takes hours for peripheral DCs to migrate to draining lymph nodes where naive T cells are resident. In addition, the interactions between DCs and T cells are thought to occur over the following 48 h or more [12]. Consequently, it remains unclear whether the differences in the allergen uptake and degradation efficiency could affect the subsequent interactions between DCs and T cells. It is also notable that the blockage and interruption of the DC/T cell interactions, e.g. cell apoptosis, could inhibit the development of effective T cell responses [25, 26]. Glycosylated proteins are reported to be cytotoxic to basophils [17]. Therefore, we evaluated the cell cytotoxicity of glycosylated BLG. As shown in Fig. 5.3B, cell viability upon stimulation by glycosylated BLG did not significantly differ from that by nonglycosylated BLG. This suggests that the enhanced uptake of glycosylated allergens may not cause a reduction in cell viability, and hence uptake is unlikely to be negatively affecting peptide presentation to T cells.

Conclusion

Glycation under extensive heating conditions changes the allergenicity and uptake of BLG by DC. Compared to nonglycated BLG, glycated BLG inhibited the degranulation of basophils in a dose-dependent manner. Glycation enhanced the uptake of BLG by DCs. The degradation of glycated BLG occurred more efficiently in DCs, but it remains to be elucidated whether these changes contribute to the altered interactions between DCs and T cells. These results indicate that extensively dry heated BLG may be less allergenic than nonglycated BLG.

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Chapter 6

General discussion



Incorporation of extensively heated (baked) milk in the diets of cow's milk allergic subjects may represent an alternative approach of oral immunotherapy (OIT) [1]. Compared to strict avoidance of allergens and OIT in the traditional sense with gradually increasing amounts of native allergens, this alternative method could be beneficial for allergic subjects to broaden their diets and improve their quality of life. Clinical studies that focused on this topic started from egg allergy and extended to cow's milk allergy (CMA) [2]. Limited but promising results were obtained and raise the need for further studies on the underlying mechanism. First, analyses of the physicochemical and immunological properties of advanced glycation end products (AGEs) that are formed during the preparation of baked milk are insufficient. Second, the mechanism underlying the observation that baked milk can accelerate the development of immune tolerance remains unclear. The objective of this thesis is to provide better understanding on these questions, based on the research as presented in the former chapters. The main findings of this dissertation are discussed in this chapter.

Impact of extensive heating on the physicochemical properties of whey proteins

"Baked milk" is described as the milk materials (milk powder or whey powder) present in baked products, such as muffins and cookies. In these foods, some parts of the material turn into a semi-dry or dry state after reaching temperatures higher than the evaporation temperature of water. In this process, water activity (a_w) and temperature are dynamic and vary with the position within the product during the baking process. To simplify and reproduce this bakery process, a_w values of 0.23 and 0.59 were chosen in this study. A_w 0.23 corresponds to the a_w of commercial milk/whey protein powder and is considered as a low water activity, characteristic of dry bakery products [3, 4], whereas $a_w = 0.59$ mimics the intermediate a_w in some semi-dry baked products [5]. In Chapter 3, pH 5 and pH 7 were chosen to cover the diversity of commercial whey products and extended to pH 9 since a high pH is known to change the protein unfolding process, certainly in combination with high temperature. In aqueous solution, pH is used as an indicator of H^+ ion activity. In a dry semi-solid state, pH is not well defined. The value as used throughout this thesis

represents the pH of the solution prior to lyophilisation. It is termed the “apparent pH” of dry materials and an abbreviated designation, “pH”, has been used [6].

During dry and semi-dry heating, glycation may increase the amount of H^+ ions as a consequence of the formation of acids, the conversion of basic amines to other compounds, and the condensation reaction between free amines of proteins and reducing sugars [7]. A greater decrease in the initial pH could be expected in the samples with a greater extent of Maillard reaction after heating [8]. Therefore, there is a relation between the “pH” of a solid material and the initial pH of the solution prior to lyophilisation, as well as its extent of glycation when dry heated. For instance, the deamidation rate of peptides and proteins during dry heating was reported to be sensitive to “pH” [9-11]. In addition, “pH” can dictate to which extent the Maillard reaction would be affected by other effectors, such as a_w and temperature [9]. A limited number of studies focused on the effects of extensive heating at such dry or semi-dry conditions [12, 13]. These previous studies made clear that the modifications in the properties of milk proteins during dry heating could not be extrapolated from results obtained in a wet system. Therefore, the first part of this research focused on the influence of dry heating on the properties of whey proteins in the presence of lactose to induce the Maillard reaction.

Formation of aggregates during dry heating

Results from chapter 2 and 3 suggest that “pH” and a_w affect the formation of protein aggregates during dry heating. Size exclusion chromatography and particle size distribution were determined to evaluate how aggregation and Maillard reaction induced changes in particle size. Results showed that the formation of large aggregates was favoured at $a_w = 0.23$ (Fig. 1.2, Fig. 2.6, Fig. 3.1) and at higher “pH” values (Fig. 2.6). In the presence of lactose, not only the aggregation of proteins, but also the Maillard reaction and the interactions between glycated proteins contributed to aggregation and the increased particle size. Elsewhere, lactose was also reported to slow down the unfolding of proteins and the subsequent aggregation at low temperature, whereas the aggregation rate is no longer dependent on the concentration of lactose when the heating temperature is above 100°C [14]. In the

experiments described in this thesis, where heating was done at 130°C, no protective effect of lactose against unfolding would thus be expected.

The fraction of PBS soluble aggregates increased with increasing “pH” (Fig. 2.6). Previous research showed that at lower “pH”, the intermolecular disulphide bonds between aggregated proteins predominated, while covalent cross-links other than disulphide bonds were also formed at higher “pH” values [12]. In addition, results in this thesis demonstrated that the Maillard reaction rate was enhanced at higher “pH” (Fig. 2.5, shows the increased formation of sRAGE-binding ligands). These two aspects can explain the increased particle size at higher “pH”.

Insoluble aggregates resulting from dry heating have been rarely studied. The formation of insoluble aggregates is sugar type- and pH-dependent. In wet conditions, glycation of BLG with arabinose and ribose at pH 4 induced the formation of much more insoluble material than the glycation with galactose, glucose, lactose or rhamnose [15]. Dry heating of whey proteins at higher “pH” values induced a greater amount of insoluble aggregates [12], whereas the heating of BLG in solution at pH 5 (near its pI) favoured the formation of insoluble material as compared with pH 7. In addition, protein glycation in solution may affect the solubility of proteins in a pH-dependent manner [16, 17]. BLG possesses enhanced stability against unfolding at acidic pH [18], except for pH values near its pI, retarding thermal-induced exposure of buried lysine. Consequently, more glycation induced insoluble aggregates would be formed after heating at higher pH. In alkaline conditions, also the browning was greatly accelerated, which can also contribute to the increase in the amount of insoluble aggregates [19]. As shown in Fig. 6.1, an increase in the amount of insoluble aggregates with increasing pH was detected in the samples with a_w 0.23 and 0.79. More insoluble material was formed in samples heated at “pH” 5 and a_w 0.59 than at pH 7. This may result from the stronger influence of this near-pI pH on the aggregate formation at this semi-dry state.

Denaturation of whey proteins upon extensive dry heating

Compared to denaturation of whey proteins heated in aqueous solution, denaturation during dry heating is largely retarded by the substantial decrease in molecular motion [20]. In addition, the rate of denaturation of whey proteins during dry heating is also

“pH”-dependent. Results in chapter 2 suggest that the denaturation rate increased with increasing “pH” (Fig. 2.2B), which is line with the result of Gulzar et al. [12].

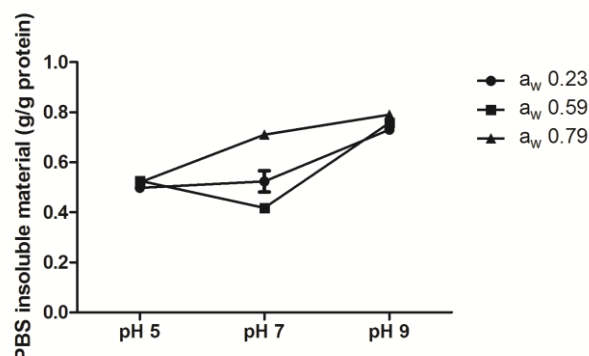


Figure 6.1. Formation of PBS insoluble aggregates from whey proteins after heat treatment in the presence of lactose at 130°C for 10 min as a function of “pH”. Data is from two independent experiments.

Denaturation is any non-proteolytic modification of the unique structure of a native protein, giving rise to definite changes in chemical, physical, or biological properties [21]. Generally, denaturation is characterised by losses in the secondary, tertiary, and quaternary structures. As shown in chapter 3, a slight increase in the content of β -sheets with prolonged heating was observed, indicating that the aggregation and formation of AGEs occurred simultaneous to the denaturation of whey proteins. In the meantime, however, native-like aggregates consisting of elements with preserved global fold and flexibility, native-like global shape, and size-related parameters may be formed [22, 23]. These elements are mainly glycated proteins. Glycation induces chemical modifications on lysine side chains of these proteins without altering the secondary and conformational structure of proteins, but changing the protein charge, driving the formation of native-like aggregates [24]. Compared to the heating conditions of these studies, a lower a_w and higher temperature (130°C) condition was applied to the dry heating system in this research. In the study of Zhou et al., the denaturation temperature of whey protein isolate (WPI) at a_w 0.23 was $163 \pm 1^\circ\text{C}$, whereas the aggregates can be formed at much lower temperature [20]. Thus, their study indicated the possibility of the formation of native-like aggregates during dry heating at low a_w . There is no report on the denaturation temperature of whey protein in the presence of reducing sugar under such heating conditions, but a relatively lower denaturation temperature could be expected because of the involvement of the Maillard reaction between proteins and reducing sugars. Therefore, it can be

speculated that dry-heating-induced aggregates consist of both denatured and native-like proteins in the presence of lactose. A decrease in the amount of BLG monomer was observed with increasing “pH” (Fig. 3.2B), and the amount of large aggregates that cannot enter the SDS-PAGE gel increased with the increase of “pH”. Both the increased large aggregates and the loss in native monomers cannot directly reflect the denaturation of whey proteins, since the aggregates may consist of native-like elements as discussed above.

Digestibility

Because the hydrophobic segments, which are more susceptible to gastrointestinal (GI) digestion, are buried inside the protein molecules [25], major whey proteins are relatively resistant to gastric (pepsin) digestion in their native state [26]. Heat treatment may expose the hydrophobic amino acids to proteolytic enzymatic action, thereby increasing digestibility. In the presence of reducing sugars, however, the reaction between amino groups and carbohydrates and intramolecular reaction between amino acids within the protein molecule retard the enzymatic hydrolysis [27]. In addition, the reduction in protein solubility caused by glycation can hinder the access of enzymes to the protein. All of these factors determine the final digestibility of dry heated whey proteins. In chapter 3, the gastric digestibility of whey proteins was found to be positively correlated to the level of unfolding of proteins as reflected by the amount of accessible amino acids, as well as the protein solubility, which is affected by glycation. Insoluble material was not separated from the PBS soluble part before the samples were digested *in vitro*. The digestibility was calculated based on the total protein of the mixture of insoluble and soluble materials, rather than only on the soluble proteins. Therefore, the solubility of glycated samples could be an important factor influencing the digestibility. Heat treatment at different water activity and “pH” conditions did not result in a significant difference in duodenal digestibility of whey proteins, except for the samples with a_w 0.59 at “pH” 9. High resistance to GI digestion of the thermal-induced precipitate in this sample may be responsible for the lower digestibility, with 90% of the precipitate in these samples still being insoluble after duodenal digestion (Fig. 6.2). Compared to the digestibility of unheated proteins [28, 29], the digestibility of dry heated whey proteins was largely reduced in all the samples prepared.

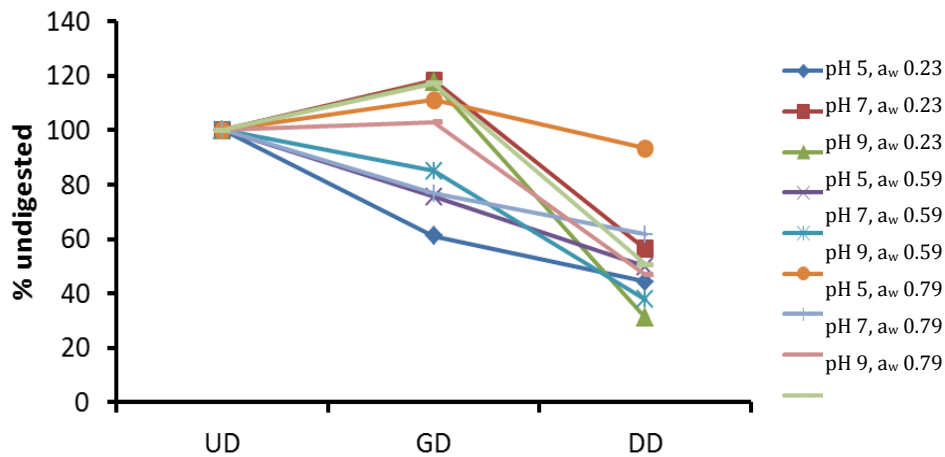


Figure 6.2. Influence of gastric digestion (GD) and duodenal digestion (DD) on the solubility of precipitates formed during dry heating.

After GI digestion, about 50% of the insoluble material became solubilised. The precipitates of the samples prepared at “pH” 5 were more readily hydrolysed by pepsin than the samples heated at higher “pH” values (Fig. 6.2), whereas more insoluble proteins were observed in the samples prepared at higher “pH”, as discussed earlier in this chapter (Fig. 6.1). As a result, more precipitates in samples with higher “pH” can be detected after gastric digestion, this may partly contribute to the decreased gastric digestibility of the samples prepared at “pH” 9 and higher a_w (Fig. 3.3). The properties of insoluble material formed during extensive heating have rarely been studied, but they may be of importance in food allergy since the 20 min dry heating rendered most of the proteins insoluble (Table 2.1). The reduced solubility of food allergens also decreases the accessibility of allergens to the immune system. However, difficulties to perform experiments on proteins in the solid state make the study on the functionality of insoluble proteins burdensome. In this research, we solubilized the heated samples in PBS pH 7.4, which can protect the protein structure during the storage and thawing of the samples, and most of studies in this thesis focused on the PBS soluble part rather than the insoluble materials. We did not use

detergents for further solubilisation of the precipitates because this process may affect the structure and functionality of proteins. Protein solubilisation by means of enzymatic hydrolysis can mimic real GI digestion in the human body, but further studies are needed to clarify to what extent the insoluble protein can be solubilized by digestion and whether the functionality of the resulting proteins differs from the PBS soluble proteins.

As CMA mainly affects infants and young children, an infant GI digestion model was used in this study. Compared to an adult digestion model, the levels of protease, phosphatidylcholine, and bile salts were reduced in this infant digestion model according to a previous report by Dupont et al. [30]. As a consequence, more “immunologically intact” proteins or peptides gain access to the immune system and immune responses were subsequently elicited. Interestingly, there were differences in cleavage patterns of peptides among the samples heated at different “pH” (Fig. 3.4A), whereas different water activity of the samples did not result in differences in peptides (Fig. 3.4B). The study of Meltretter et al. demonstrated that both heating condition and sample composition affect the glycation sites [31]. Thus, digestion of these glycated proteins may result in different peptides, as observed in this research (Fig. 3.4A), due to differences in glycation sites.

Formation of sRAGE-binding ligands

Heating conditions, including nutrient composition, temperature, heating time, water activity, and pH of the food matrix, affect the generation of AGEs during heating [32]. Extensive dry heating conditions, 130°C at a_w 0.23 and 0.59, were applied in this research. Substantial amounts of AGEs formed during heat treatment of whey protein/lactose or BLG/lactose can be expected under such conditions. As AGEs are potentially involved in the aetiology of several diseases, such as diabetes, and some kidney disease [33], the formation of AGEs in extensively heated foods could be considered as an undesired effect towards their immune tolerance-inducing effect. However, it is possible that AGEs, at least partly, are responsible for the influence of baked milk on the induction of immune tolerance. Thus, a balance between reduction of the undesired effects and preservation of the beneficial effects of glycated proteins

to immune tolerance should be pursued in the future before baked milk can be used in clinical practice.

In chapter 2 and chapter 3, we investigated the formation of AGEs and the factors affecting their formation. Results suggested that “pH” and a_w affect the rate of formation of AGEs. Less sRAGE-binding ligands were detected in samples with “pH” 5 than in samples with higher “pH” values (Fig. 3.5). In solution, the rate of Maillard reaction is reported to be low at acidic pH, increasing with increasing pH until the maximum is reached at pH 10 [34]. Similar results were obtained in our dry and semi-dry heating system. This can be explained by the pH dependence of the formation of reactive dicarbonyls via the Namiki pathway, as discussed in chapter 3. The Maillard reaction rate is also carbohydrate-dependent, making the results from different studies sometimes not comparable [15, 19]. However, it is generally acknowledged that alkalinity favours the Maillard reaction.

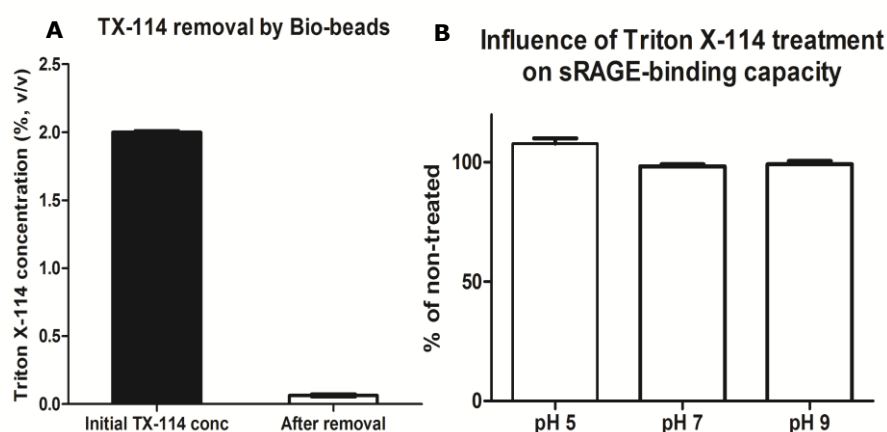


Figure 6.3. Influence of LPS removal on the sRAGE-binding capacity of glycated samples. TX-114 was removed by bio-beads and the remaining A: TX-114 concentration was determined by spectrophotometer using absorption at 280 nm. B: the influence of the LPS removal procedure on the sRAGE-binding capacity of BLG samples was tested by an ELISA-based sRAGE-binding assay. Error bars represent three independent measurements.

It should also be taken into account that, before cell experiments can be performed, the lipopolysaccharide (LPS) should be removed from the samples. To study whether the LPS removal procedure affects the properties of AGEs and their subsequent roles in interactions with cells, the influence of LPS removal by triton X-114 (TX-114) on the sRAGE-binding capacity of heated samples was determined. As shown in Fig. 6.3B, no significant differences in sRAGE-binding capacity were observed between the TX-114 treated and non-treated samples, indicating that the LPS-removal procedure did not interfere with the AGE-sRAGE interaction.

Impact of extensive heating on the immunological properties of whey proteins

Immunoreactivity

The IgG-binding capacity of glycated whey proteins was evaluated in chapter 2. The IgG-binding capacity of allergens reflects the immunoreactivity, but it does not necessarily reflect the ability of allergens to elicit an allergic response. Similar to IgE, IgG is able to bind conformational and sequential epitopes of allergens, and there normally are overlaps between IgE and IgG epitopes. Wang et al. reported that IgE epitope diversity correlated with persistence and severity of CMA, whereas there was no correlation between the number of IgG4 epitopes and severity of allergic reactions [35]. Another study reported that interleukin (IL)-4 secreted by Th2 cells induces both IgE and IgG4 switching in B cells, while the immunomodulatory cytokine IL-10 inhibits IgE production but enhances IgG4 production [36]. In addition, the IgE/IgG4 ratio is reported to be important in the prediction of reactivity and tolerance development [1, 37, 38].

The mechanism how IgG4 is involved in type 1 food allergy and induction of immune tolerance remains unclear, but it is clear that IgG plays a role in these processes. A decrease of 67% in IgG-binding capacity compared to unheated samples was observed. The denaturation and glycation-induced destruction of IgG epitopes, may negatively affect the IgG-binding capacity, whereas exposure of some initially buried epitopes and formation of new epitopes (neoepitopes) may enhance the binding capacity. Fig. 2.4 shows that 67% and 80% of their lysine residues are located in the epitopes of α -

lactalbumin (ALA) and BLG, respectively. The reaction between lysine and carbohydrate during glycation induces steric hindrance and destruction of epitopes and subsequently reduces the IgG-binding capacity of ALA and BLG. Furthermore, the aggregation of proteins may also hinder the binding of whey proteins to IgG, e.g. by shielding of epitopes. Interactions between IgG and allergens may not directly lead to an immune reaction that contributes to type 1 food allergy, but the same epitope of an allergen might be bound by both IgG and IgE antibodies. These IgG antibodies were reported to be involved in CMA by means of binding to IgE receptor-expressing cells, such as mast cells and basophils [39], and the competition between IgE and IgG antibodies at the antigen-presenting stage has also been reported [40]. Therefore, isotypes of IgG (IgG1 in mice, IgG4 in human) play important roles in CMA, and the decreased IgG-binding capacity indicates the ability of glycation to modulate the functionality of whey proteins.

The IgE-binding capacity of glycated whey proteins was evaluated in Chapter 4. Similar to IgG binding, the IgE-binding capacity of glycated allergens is allergen type and heating condition-dependent. Destruction of conformational epitopes can lead to a decrease in the accessibility of linear epitopes, and therefore lead to a decrease in the IgE-binding capacity of glycated allergens. The IgE-binding capacity may however also be enhanced by the formation of neoepitopes that result from thermal modification of allergens [41], and the exposure of epitopes initially buried inside the protein molecules. Analyses similar with that presented in Fig. 2.4 have been performed. Results revealed that most lysine residues were located in the IgE epitopes of BLG and ALA. Hence, the glycation occurring between lysine and lactose could lead to both the destruction and formation of epitopes, and therefore both a decrease and increase in the IgE-binding capacity could take place.

A higher IgE-binding capacity of glycated allergens was observed as tested by dot-blot (Fig. 4.3B). This could be explained by the fact that IgE, like the scavenger receptors, has a specificity for the anionic character and alkylated lysine provided by AGEs [42, 43]. The increase in IgE-binding capacity was contributed to by the interactions between the anionic character and alkylated lysine of AGEs rather than the reaction between epitope and IgE antibody, thus it cannot initiate the allergic responses of mast cells and basophils (Fig. 5.1). Our data did not exclude the possibility that the

formation of neoepitopes could also have contributed to the increased IgE-binding capacity. As there are overlaps between IgE and IgG epitopes [40], however, the formation of neoepitopes would possible also have an increase in IgG-binding capacity, which was not observed in our study (Fig 2.5). Therefore, it can be hypothesized that neoepitopes did not solely determine the increased IgE-binding capacity.

Macrophage immunogenicity

Functional tests on immune cells, such as basophils, macrophages, dendritic cells, and mast cells, were done by other researchers to evaluate the immunological properties of AGEs [44, 45]. In this research, we evaluated the macrophage immunogenicity of glycated BLG in chapter 4. Results showed that glycation of BLG reduced the polarisation of macrophages towards the pro-inflammatory M1-phenotype, and glycation was capable of inducing the formation of components with potential anti-inflammatory properties as tested with M0, M1, and M2 macrophages. Induction of immune tolerance is a complicated process, but as introduced in the general introduction of this thesis, the interactions between T cells and antigen presenting cells are essential in this process. Therefore, the results in chapter 4 and chapter 5 (Fig. 5.2) demonstrated a potential role of the antigen presenting cells (macrophages, dendritic cells) in the induction of immune tolerance induced by extensively heated milk, by enhancing the secretion and gene expression of immunomodulatory cytokines.

The macrophage immunogenicity of glycated proteins is correlated to their physicochemical properties as modified by extensive dry heating. It was reported that the phagocytosis of microspheres by macrophages is molecule size-dependent [46]. A maximal phagocytosis was detected at the size range of 1.0-2.0 μ m. As shown in Fig. 2.2, for the samples heated for 10 min, no difference in the percentage of aggregates with particle diameter of 1.0-2.0 μ m can be observed between the samples at a_w 0.23 and 0.59. However, a higher amount of large aggregates, which may possess suppressed phagocytosis efficiency [46], was found in the samples at a_w 0.23 than at 0.59 (Fig. 4.1), potentially resulting in a lower uptake efficiency via phagocytosis of proteins from the samples at a_w 0.23. Thus, enhanced uptake of glycated proteins at a_w 0.59 via phagocytosis by macrophages may be related to their enhanced

immunomodulatory properties, as observed in Fig. 4.5. A higher uptake efficiency of samples at a_w 0.59 by another APC, dendritic cells, was also detected (Fig. 5.2). This increased uptake by DCs may also be related to the size of the proteins.

Potential contribution of dry heated milk to the development of immune tolerance

Multiple immunologic determinants, including APCs, T cells, cytokines, antibodies [47], effector cells (basophils and mast cells), and vitamin D [48], play essential roles in shaping the tolerogenic responses. This research tested the influence of extensively heated whey protein on antibodies (chapter 2 and chapter 4) and APCs (chapter 4 and chapter 5), and the subsequent consequences (polarization, anti-inflammatory cytokine secretion) that may be involved in the induction of immune tolerance. Preliminary results from this research indicate a potential role of glycation/AGEs in inducing immune tolerance, but no real conclusion can be drawn until experiments on Tregs and *in vivo* studies have been performed.

In chapter 5, enhanced uptake and degradation of glycated BLG by dendritic cells were reported. Affinity of the mannose and other lectin-like receptors for the carbohydrate domains, and increased size of glycated proteins, may be responsible for the increased uptake rate. The mechanism and speed of the allergen uptake by APCs, and the compartment in which the antigen accumulates, might direct the course of the induced immune responses [49]. Burgdorf et al. showed that AGE-OVA with uptake via mannose receptor was only presented to CD8⁺ T cells, while pinocytosed AGE-OVA was presented to CD4⁺ T cells [50]. In addition, AGEs change the maturation, phenotype, and function of DCs themselves in a dose-dependent manner [51], thus the enhanced uptake of glycated allergens by DCs may alter the cytokine production of DCs already before they encounter T cells. It takes hours for peripheral DCs to migrate to draining lymph nodes where naive T cells are resident, and the interactions between DCs and T cells are thought to occur over the following 48 h or more [52], also making modification of DC properties by glycated allergens prior to interactions with T cells possible.

Glycated BLG showed a reduced allergenicity as tested by the degranulation assay of basophils (Fig. 5.1), and enhanced degranulation efficiency of glycated BLG was

detected. These results are in line with the result in chapter 4, which also indicated that the protein glycated at a_w 0.59 possesses an enhanced immunomodulatory property. It is notable that less AGEs were generated in the samples heated at a_w 0.59 (Fig. 2.6, Fig. 3.5), indicating that not only AGEs, but also other Maillard reaction products or heat-induced non-Maillard compounds in protein contribute to the immunomodulatory properties of glycated protein. Less formation of AGEs suggests the possibility that more products in the early stage and/or the final stage of the Maillard reaction are generated. This is confirmed by the result shown in Table 1.1, where more furosine can be detected in the samples heated at a_w 0.59 for 10 min. Therefore, data in this thesis indicates an important role of AGEs in conferring immunomodulatory properties to glycated proteins, but it indicates that other Maillard reaction products or heat-induced non-Maillard compounds possibly contribute as well.

The presence of IL-10 may be essential in the induction of immune tolerance, by exerting immunomodulatory properties on both the immature DCs and T cells. IL-10 is capable to inhibit the development of fully mature DC and converts immature DCs into tolerogenic APC [53]. Interactions between IL-10 and its receptors induce signals that maintain immature DCs in their immature state even in the presence of maturation signals [54, 55]. With regard to T cells, the tolerogenic DCs (tDCs) provide stimuli that may act directly on T cells and/or modify environmental conditions facilitating the induction of Treg cells. Among these stimuli, the secretion of IL-10 by tDCs is essential for maintaining the tolerogenic properties of Treg cells in a variety of models of Treg differentiation [56]. In turn, Treg cells secrete IL-10 contributing to the maintenance of tolerogenic attributes of tDCs.

Preliminary results on the mechanism of dry heated milk induced immune tolerance have been obtained in this study. The characteristics of glycated allergens were evaluated, and indications for why baked milk can be consumed by many cow's milk allergic children, and why it contributes to the development of immune tolerance were obtained (Fig. 6.4). Further studies are needed to test some hypotheses based on this study (dashed part in Fig. 6.4).

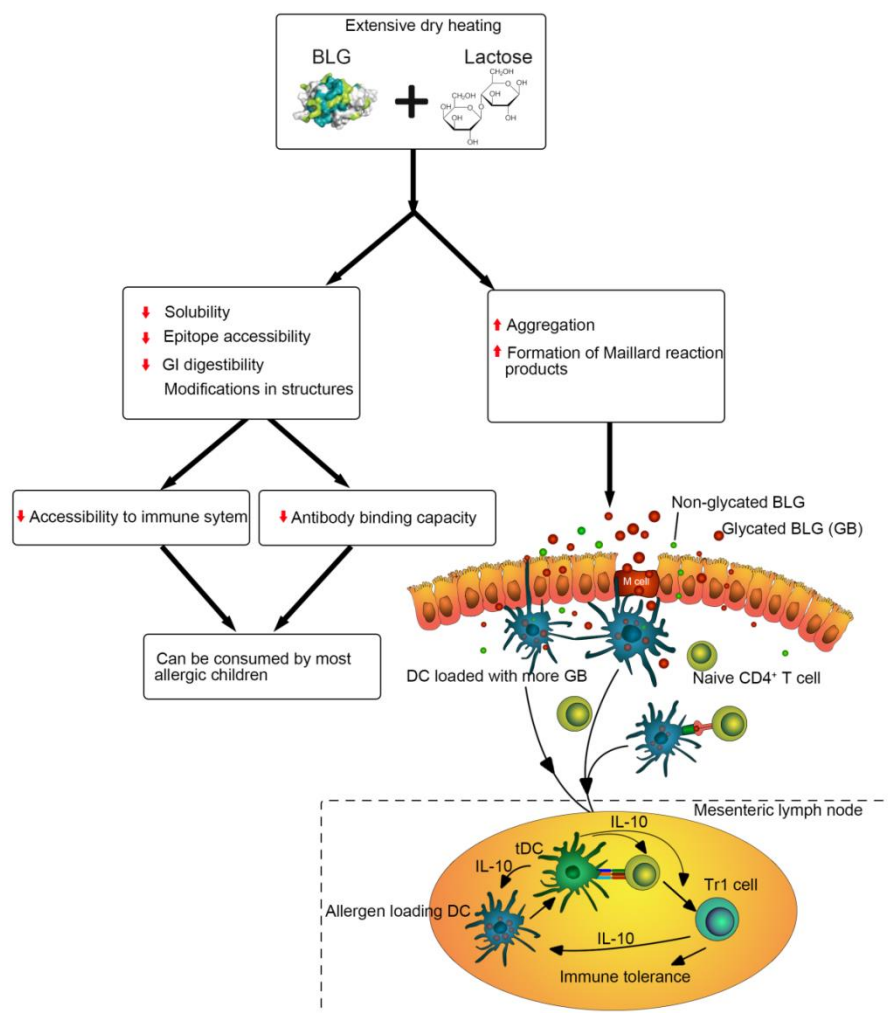


Figure 6.4. Potential contribution of dry heated BLG to the development of immune tolerance based on the results in this research. Dry heating induces modifications in the properties of BLG, decreasing the availability to immune system and antibody-binding capacity of BLG. Aggregated and glycated BLG is more efficiently taken up by immature dendritic cells. The interactions between thermal-modified BLG loaded DCs and naive CD4⁺ T cells render the DCs into tolerogenic DCs (tDCs). tDCs stimulate the generation of Tr1 cells via an IL-10 involved mechanism. Tr1 cells in turn contribute to the generation of tDCs from BLG loaded DCs. Hypothesis based on our results in this research was proposed in dashed box.

Future perspectives

This thesis demonstrated that extensive dry heating induced profound effects on the protein properties, such as structure, size, aggregation, denaturation, and immunoreactivity, conferring immunomodulatory properties on glycated proteins as tested in macrophages. The presented data indicated that heating conditions affect the outcomes of extensively dry heating. First, the effects of water activity and “pH” were evaluated. To extend this study, more factors, such as carbohydrate type, the presence of lipids [57], and other milk proteins (especially caseins) should be taken into account in the preparation of extensively heated allergens. Second, in chapter 3, differences in enzymatic cleavage patterns of peptides among the samples heated at different “pH” were detected. Due to the complexity of peptides resulting from protease hydrolysis, our work and most of other researchers’ work on the immunological properties of glycated allergens mainly focused on non-hydrolysed proteins. As most of the ingested allergens are absorbed and presented to immune cells by humans in the form of peptides, studies on the performances of glycated peptides in inducing immune responses are needed in the future. Third, potential roles of glycated allergens in the development of immune tolerance were suggested in this research, but their effects on the generation of Treg cells and *in vivo* development of immune tolerance were not studied. The development of immune tolerance to cow’s milk allergens is a complicated process, thus overall studies with the involvement of more factors and mechanisms need to be conducted.

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Summary

Summary

Baked milk products, e.g. milk-protein containing muffins or baked cheese, can be tolerated by most cow's milk (pasteurized milk, infant formula, etc.) allergic subjects. These products were also reported to contribute to the development of immune tolerance in allergic subjects, thus their incorporation into the diets of allergic people is considered as an alternative approach of oral immunotherapy in several clinical studies. Observational results from these studies not only indicate a promising future for the use of baked milk in the management of cow's milk allergy (CMA), but also raise the need for further studies on the consequences of extensive dry heating on milk proteins. The main objective of this thesis was to investigate the effects of heating under dry conditions on the physicochemical and immunological properties of whey proteins.

In **Chapter 2**, the changes in the properties of whey proteins under controlled dry heating conditions were studied. A simplified heating model, consisting of whey proteins and lactose, was used to reproduce baking conditions. Properties of allergens that may affect their ability to induce immune responses, such as solubility, exposure of SH groups, loss of available lysine, aggregate size, and IgG-binding capacity, were studied. The formation of advanced glycation end products (AGEs) was tested by an sRAGE-binding assay. The dry heating was accompanied by a loss of soluble proteins and an increase in the size of dissolved aggregates. Most of the Maillard reaction sites were found to be located in the reported conformational epitopes on whey proteins. Therefore, the structural changes, including exposure of the SH group, SH-SS exchange, covalent cross-links, and the loss of available lysine, subsequently resulted in a decreased IgG-binding capacity. The binding of glycation products to the receptor of AGE (RAGE) increased with heating time, which was correlated with the stage of the Maillard reaction and the decrease in IgG-binding capacity. The RAGE-binding capacity was higher in samples prepared at water activity (a_w) 0.23. These results indicate that intensive dry heating of whey proteins, as it occurs during baking, may influence the immunological properties of allergens in cow's milk, both due to chemical modifications of the allergens and formation of AGEs.

The formation of AGEs especially occurred at extensive dry heating and was affected by a_w of samples during preparation. In **Chapter 3**, the formation of AGEs was further

studied. Differences in cleavage patterns of protein into peptides among samples heated at different “pH” values, which is the pH of the solution prior to lyophilisation, were observed. Formation of sRAGE-binding ligands depended on the aggregation, “pH”, and a_w of the samples. Moreover, the sRAGE-binding activity of the samples after digestion was changed and correlated with the digestibility of samples. These results suggested that the generation of sRAGE-binding ligands during dry heat treatment is dependent on heating conditions, and correlated to the properties of extensively dry heated proteins, such as the digestibility, solubility, and aggregation.

In **Chapter 4**, the macrophage immunogenicity of glycated BLG was studied. A correlation between the formation of AGEs and their immunogenicity was hypothesized. The IgE-binding capacity of glycated samples and their influence on the polarization and gene expression of macrophages were studied in vitro. Glycation of BLG was found to reduce the expression of pro-inflammatory TNF- α , and increase the expression of anti-inflammatory TGF- β in M1 and M2 macrophages. These results indicate that glycation of BLG under controlled temperature and water activity conditions may retard polarisation of macrophages towards the pro-inflammatory M1-phenotype. The BLG samples glycated at a_w 0.23, in which more sRAGE-binding ligands were formed, were more efficiently enhancing the expression of anti-inflammatory cytokines and reduced the expression of the proinflammatory cytokine TNF- α . These results indicate that not only AGEs, but also other Maillard reaction products might be responsible for the potential immunomodulatory function of dry heated, glycated BLG.

The immunomodulatory potential of glycated BLG was further studied, as described in **Chapter 5**. This chapter continued the work in chapter 4 by studying the responses of immune cells in both the sensitization phase and effector phase of food allergy due to the stimulation by glycated BLG. The uptake of glycated BLG by dendritic cells (DCs) was studied. The study on the basophil responses to glycated BLG was also included in this chapter. In line with the results in chapter 4, results in this chapter showed that glycated BLG inhibited the degranulation of basophils in a dose-dependent manner. Glycation of BLG enhanced its uptake by DCs. However, the degradation of glycated BLG was faster than unheated BLG, indicating a retarded allergen-presentation efficiency of glycated BLG by DCs. These results indicate that extensively dry heated BLG may be less allergenic, and is processed differently by DCs, than nonglycated BLG.

Chapter 6 discussed the contribution of glycated whey proteins on the induction of immune tolerance based on the results described in chapters 2-5. A relation between the changes in the properties of BLG during heating and the immunological consequences was proposed. The main conclusions and recommendations for future research were presented in this chapter. A mechanism to explain development of immune tolerance induced by extensively heated whey proteins was hypothesized.

In conclusion, this thesis showed that extensive dry heating induces profound and specific effects on the physicochemical and immunological properties of whey proteins. Conditions during heating, such as a_w and "pH", affect the consequences of heating on whey proteins and their subsequent functions in interacting with immune cells. The cleavage patterns of proteins after in vitro digestion was different for the samples heated at different "pH", affecting the sRAGE-binding activity. Compared to unheated and nonglycated samples, glycated BLG can be more efficiently taken up and degraded by DCs. In addition, glycation confers immunomodulatory properties on whey proteins, as tested in macrophages. These results might have consequences for preparing extensively dry heated allergens that can be used in oral immunotherapy. The data in this thesis also provided a better understanding on the mechanism underlying the observation that the development of immune tolerance can be accelerated by baked milk.

Acknowledgements

Acknowledgements

Doing a doctoral research was a wonderful journey, during which many great people helped and inspired me. I've had ups and downs in this journey, all made the past five years colourful and memorable. The end of this journey is the beginning of another one. I don't know what I will confront in the coming journey, but I am confident to expect a promising future accompanied by the beautiful memory with these lovely people here.

I would like to thank my supervisor and co-promotor Kasper Hettinga. Kasper, it has always been a great pleasure to work with you. Discussions with you were always inspiring. You can easily get to the right point even if I express my ideas in a vague way, this was really helpful especially at the beginning of my PhD study. I am impressed by your efficiency in work. I could always get your prompt responses to my manuscripts and reports. I learned loads from you, how to start a scientific plan, how to be critical and structured in my thinking, and how to extract useful information from the results I obtained.

I gratefully acknowledge my promotor Tiny van Boekel. Tiny, thanks for being critical and nice in all our progress meetings. During our PhD trip to UK, I still remember you were so thoughtful and kind to encourage me, and give me many advices on my first presentation. Your experience and wisdom could always help me a lot in writing and analysing data. You could always convince me and put things into the right direction, especially in the writing of my thesis. Thanks you so much!

I would like to thank my promotor Harry Wichers. Dear kind-hearted, gorgeous Harry, thanks for your good sense of humour to make all the scientific talks not boring. Thanks for your help in obtaining the sera samples and doing experiments in FBR. I like the straightforward way to discuss with you, and also your unprofessional but clear drawing for interpreting your ideas during our progress meetings.

I am very thankful to my co-promotor Gosia Teodorowicz! Gosia, I appreciate and greatly value your advices. I enjoyed working with you. Your help in sRAGE experiments largely contributed to my articles. The protocol you made can be easily followed. The statistical software you recommended helped me a lot. Thank you so much for all your support!

I would like to thank all BSc and MSc students who were partially involved in my project. Meng Wang, Xinyu Li, Liske Henken, Marjolein Marks, Man Su, Yuli Liang, Marios Voulgarelis, Charlotte Hendriks, Liyou Dong, thanks for all your efforts, great contribution and valuable outcomes.

I am sincerely grateful to Jenny Hallgren Martinsson and Behdad Zarnegar in Uppsala University. Thanks a lot for your help during my three-month study in Uppsala. I really enjoyed the atmosphere in your group. Cell experiments were totally new to me at that moment, but I have learned a lot under your patient supervision. Behdad, you are really a good teacher! I wish you all the luck with finishing your thesis! Also, many thanks to Shanna Bastiaan-Net in the FBR group for helping me with cell experiments and dot-blot analysis. Your contributions to my last two papers are appreciated! I also would like to thank Joost Smit from Utrecht University for helping me with the cell experiments. I enjoyed our discussion on the experimental plans and the manuscript.

I would like to thank the PhD students and staff in FQD. I will keep the great memories about our coffee breaks, PhD-trips, lab-trips, lunches, and Christmas dinners. I feel honoured to work with you in this great group. Special thanks to Chunyue Zhang and Moheb Elwakiel for being my Paranympths.

I am sincerely grateful to Prof. Hongbing Chen. Thanks for recommending me to study in this fantastic group. I still remember you showed me some slides to introduce the research and life in Wageningen University, based on your one-year stay here as a visiting scholar. That presentation inspired me to seek for a PhD position in Wageningen University to continue my work. I got support from you during the preparation for applying the PhD position. You even prepared a headset for me for the Skype interview. During my PhD study, we discussed now and then on life and research. Chatting with you can always help me out of problems, motivate me to explore new territories, and inspire me to be enthusiastic about life and work. I really appreciate all your support.

I also wish to thank my Chinese friends here who accompanied me in this wonderful PhD journey! 田灵敏, 老魏, 唐永富, 家翠, 唐静, 小蓉, 陈敏, 依丽娅, 浩峰, 石文标, 芦晶, 张丽娜, 陆伟, 金峰, 宫宝, 孙恬, 刘坤, 翔明, 程立绒, 武启蒙, 宋银, 谷方婕, 张春月, 王珏, 王娅, 邓羽西, 邱俊, 王昭君, 邹苑, 万芝力, 任映雪, 高菲菲, 严静, 刘宁晶, 邓颖, 杨宇珍, 曹家璐, 还有很多相识在瓦村的朋

ACKNOWLEDGEMENTS

友们，很庆幸能在异国他乡与你们相识相知，你们的陪伴让这段漫长枯燥的旅程变得丰富多彩。我会珍藏这段美好的回忆，憧憬将来的某一天，与你们再次相逢在世界的某一个角落，一起谈论瓦村时候的生活，会是多么美好的事情。

A handwritten signature in black ink, appearing to be 'Fulmi'.

Last but not least, I would like to thanks my family! 爸妈，姐姐，谢谢你们一直以来无私的奉献和支持，我因有这样的家人而感到自豪！岳父岳母，有你们的支持和理解，才有我们现在的生活。多宝妈，谢谢你的理解宽容和付出，和你一起慢慢变老、一起看多宝成长是多么幸福的事情！多宝，当你能看懂这段话的时候，爸爸会告诉你：有你的生活，比爸爸的这本论文还要精彩！

刘法辉

2016.11.15

ACKNOWLEDGEMENTS

About the author

Curriculum Vitae



Fahui Liu was born on April 2nd, 1985, in Hubei, China. In 2004, he started his Bachelor's degree in Shihezi University, Xinjiang, China. After graduating in 2008, he continued to study for a Master's degree in Nutrition and Food Hygiene at State Key Laboratory of Food Science and Technology at Nanchang University. He completed his MSc thesis entitled 'Molecular modelling and conformational IgG epitope mapping on bovine β -casein' under the supervision of Prof. Hongbing Chen. In 2011, he received scholarship from China Scholarship council to perform a doctoral research at Dairy Science and Technology group, FQD, Wageningen University. The PhD project is about the induction of immune tolerance induced by extensively dry heated milk proteins. The results of his PhD research are presented in this thesis.

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List of publications

Liu, F., Teodorowicz, M., Wichers, H. J., Van Boekel, M. A., Hettinga, K. A., Generation of Soluble Advanced Glycation End Products Receptor (sRAGE)-Binding Ligands during Extensive Heat Treatment of Whey Protein/Lactose Mixtures Is Dependent on Glycation and Aggregation. *Journal of Agricultural and Food Chemistry* 2016, 64, 6477-6486.

Liu, F., Gao, J., Li, X., Chen, H., Molecular modeling and conformational IgG epitope mapping on bovine β -casein. *European Food Research and Technology* 2016, 1-10.

Liu, F., Teodorowicz, M., van Boekel, M. A., Wichers, H. J., Hettinga, K. A., The decrease in the IgG-binding capacity of intensively dry heated whey proteins is associated with intense Maillard reaction, structural changes of the proteins and formation of RAGE-ligands. *Food & function* 2016, 7, 239-249.

Yuan, J., Gao, J., Li, X., Liu, F., et al., The tip of the “celiac iceberg” in China: a systematic review and meta-analysis. *PLoS One* 2013, 8, e81151.

Tong, P., Gao, J., Chen, H., Li, X., et al., Effect of heat treatment on the potential allergenicity and conformational structure of egg allergen ovotransferrin. *Food Chemistry* 2012, 131, 603-610.

Xin, L., Hongbing, C., Jinyan, G., Fahui, L., Xuefang, W., Epitope mapping and identification of amino acids critical for rabbits IgG-binding to linear epitopes on buffalo beta-lactoglobulin. *Protein and peptide letters* 2012, 19, 1103-1111.

Liu, F., Teodorowicz, M., Bastiaan-Net, S., Dong, L., De Jong, N. W., Wichers, H. J., Van Boekel, M. A., Hettinga, K. A., Controlled Maillard Reaction upon Extensive Dry Heating Potentially Confers Anti-inflammatory Properties on β -lactoglobulin as Tested with THP-1 Macrophages. (Submitted for publication)

Liu, F., Smit, J. J., Teodorowicz, M., Bastiaan-Net, S., Wichers, H. J., Van Boekel, M. A., Hettinga, K. A., Glycation of β -lactoglobulin in extensive heating condition enhances its uptake by dendritic cells and induces immunomodulatory cytokine interleukin 10 in the co-culture of dendritic cells and T cells. (Manuscript in preparation)

Overview of completed training activities

Discipline specific activities

International conferences

- 9th International Symposium on Milk Genomics & Human health (2012), Wageningen, The Netherlands
- European Academy of Allergy and Clinical Immunology Congress (2015), Barcelona, Spain

Workshop & Training

- Biorefinery for Biomolecules (2012), Wageningen, The Netherlands
- Cell culture training (2013), Uppsala, Sweden

Courses

- Statistics for VLAG PhDs (2012), VLAG/Biochemistry, Wageningen, The Netherlands
- Chemometrics (2016), VLAG/Biochemistry, Wageningen

General courses

- Information Literacy PhD including EndNote Introduction (2011), WGS, Wageningen
- VLAG PhD Week (2012), VLAG, Baarlo
- Project and Time Management (2012), WGS, Wageningen
- Interpersonal Communication for PhD Students (2012), WGS, Wageningen
- Scientific Publishing (2012), WGS, Wageningen
- Video and Audio content in Scientific Communication (2014), WGS, Wageningen

Optional courses and activities

- Preparation of Research Proposal (2011)
- DST meetings and Seminars in Food Quality and Design (2011-2015), Wageningen
- PhD Excursion (2012), Food Quality and Design, United Kingdom
- PhD Excursion (2014), Food Quality and Design, Singapore and Thailand

The research described in this thesis was conducted under scholarship granted by the China Scholarship Council (No. 2011682006).

Financial support from Wageningen University for printing this thesis was gratefully acknowledged.

Cover design by Fahui Liu

Layout by Fahui Liu

Printed by Digiforce, Vianen (NL) (www.dfprint.nl)