

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

## Toward celiac-safe foods

Investigation of the interaction between transglutaminase 2 and gluten

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INVESTIGATION OF THE INTERACTION BETWEEN TRANSGLUTAMINASE 2 AND GLUTEN

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## ABSTRACT

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Celiac disease, a chronic autoimmune enteropathy, may develop in genetically predisposed individuals upon ingestion of gluten proteins found in wheat, barley, and rye. Overall prevalence of celiac disease is increasing and it currently affects around 1% of the population. The types and severity of symptoms of celiac disease show high variability, leading to many sufferers remaining undiagnosed. The only available treatment is to follow a strict gluten-free diet, but gluten-free alternatives are less available, more expensive, and often have lower nutritional and sensorial quality.

This thesis work examined the interactions between the intestinal enzyme transglutaminase 2 and gluten peptides. Transglutaminase 2 plays a significant role in disease initiation and progression, and is the main autoimmune target in developed celiac disease. A method for measuring the interaction between transglutaminase 2 and gluten was developed and tested in studies on sourdough fermentation of wheat flour and bread. Transglutaminase 2-mediated transamidation of gluten was assayed and the extent of available binding motifs for transglutaminase 2 in  $\alpha$ 2-gliadin, considered the most immunogenic part of gluten, was assessed using an ELISA-based method. The results showed that lactic acid fermentation, which is not specifically tailored to degrade gluten, cannot sufficiently prevent transglutaminase 2 interaction with gluten or decrease the extent of available binding motifs for transglutaminase 2 on  $\alpha$ 2-gliadin. In studies investigating the possibility to block specific binding motifs for transglutaminase 2 on gluten peptides, using molecules suitable as food additives, binding to  $\alpha$ 2-gliadin was computationally simulated and promising candidates were identified. These candidates were analyzed *in vitro* for the ability to prevent transglutaminase 2-mediated transamidation and deamidation of gliadin. Ascorbyl palmitate was found to interact with  $\alpha$ 2-gliadin in computer simulations and effectively reduced gliadin interaction with transglutaminase 2 *in vitro*. The cytotoxicity profile of ascorbyl palmitate, in combination with gliadin, was evaluated in Caco-2 cell cultures by determining cell survival, direct cytotoxicity, inflammatory mediators, and cell layer integrity, and no negative effects were found. In ancillary studies of human ileostomy contents after ingestion of raw and extruded gluten-containing products, degradation products of  $\alpha$ -gliadin were identified and the effect of extrusion on digestion was investigated. Preliminary results indicate that protein digestibility was decreased after intake of the extruded product, but the effect on  $\alpha$ -gliadin digestion needs further evaluation. However, the majority of  $\alpha$ -gliadin seems to be undigested after *in vivo* digestion in both products.

The interaction between transglutaminase 2 and gluten is crucial for celiac disease and in this thesis work, several strategies for preventing this have been explored. Ascorbyl palmitate has been shown to effectively prevent this interaction *in vitro* and is thus a promising candidate for creating cereal-based foods potentially safe for celiacs.

**Keywords:** celiac disease, gluten intolerance, gluten, gliadin, transglutaminase 2, TG2, ascorbyl palmitate, zinc, Caco-2 cells, gluten digestion, ileostomy subjects, extrusion cooking

## LIST OF PUBLICATIONS

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This doctoral thesis is based on the work contained in the following papers:

- I. Niklas Engström, Ann-Sofie Sandberg and Nathalie Scheers. Sourdough fermentation of wheat flour does not prevent the interaction of transglutaminase 2 with  $\alpha$ 2-gliadin or gluten. *Nutrients* 2015, 7, 2134-2144.
- II. Niklas Engström, Patricia Saenz-Mendez, Johan Scheers, Nathalie Scheers. Towards Celiac-safe foods: Decreasing the affinity of transglutaminase 2 for gliadin by addition of ascorbyl palmitate and  $ZnCl_2$  as detoxifiers. *Scientific Reports* 2017, 7(1), 77.
- III. Niklas Engström, Ann-Sofie Sandberg and Nathalie Scheers. Cytotoxicity profile of ascorbyl palmitate-gliadin in cultured intestinal cells: Aiming for celiac-safe gluten products. *Submitted*.
- IV. Niklas Engström, Nathalie Scheers and Ann-Sofie Sandberg. A study of gluten digestion in the stomach and small intestine of human ileostomy subjects. *Manuscript in progress*.

## CONTRIBUTION REPORT

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Paper I: Niklas Engström (NE), participated in the design of the study, performed the experimental work, and wrote the draft manuscript.

Paper II: NE performed the experimental work and contributed to writing the manuscript.

Paper III: NE participated in the design of the study, performed the experimental work, and wrote the draft manuscript.

Paper IV: NE participated in the design of the study, performed the experimental work, and wrote the draft manuscript.

## ABBREVIATIONS

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AP	Ascorbyl palmitate
APC	Antigen-presenting cell
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Caco-2	Cancer coli-2, cells originating from a human colorectal adenocarcinoma
CD	Celiac disease
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
FBS	Fetal bovine serum
GFD	Gluten-free diet
GM-CSF	Granulocyte macrophage colony-stimulating factor
GRAS	Generally regarded as safe
HBSS	Hank's balanced salt solution
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
LDH	Lactate dehydrogenase
MQ	Milli-Q, ultrapure water
PBS	Phosphate buffered saline
TBS-T	Tris-buffered saline-Tween
TG2	Transglutaminase 2
TNF	Tumor necrosis factor

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# 1. INTRODUCTION

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Celiac disease, also known as gluten intolerance, is an autoimmune disorder affecting roughly 1% of the population, but the prevalence has steadily increased in recent decades (1). The disease may develop at any age in genetically predisposed individuals and is manifested after ingestion of gluten protein in wheat, or similar proteins in barley and rye (2, 3). The main genetic risk factors are the human leukocyte antigen (HLA) class II molecules HLA-DQ2 and HLA-DQ8, which present antigens on antigen-presenting cells. However, 30-40% of the European population have one or other of these molecules, but only a subset develops celiac disease, so other risk factors clearly also contribute to disease development (4). Transglutaminase 2 is an important participant in celiac disease. It is an endogenous enzyme present in the gut that is able to selectively deamidate certain gluten peptides, thus greatly increasing their affinity for the HLA-DQ2/DQ8 molecules (5). Symptoms of celiac disease can vary greatly, from very clear gastrointestinal symptoms to more or less no symptoms (6). A gluten-free diet is currently the only treatment available for celiac disease. However, a gluten-free diet suffers from several drawbacks in that e.g., gluten-free alternatives often are more expensive, less available, and may have inferior nutritional and sensorial quality (7, 8). Moreover, strict adherence is difficult in the long term and untreated celiac disease increases the risk of several complications and diseases (9, 10).

Several approaches to improve the situation for celiacs are currently being investigated. Improvement of gluten-free products is one way, either by improving the formulation of ingredients or by treatment of wheat flour with lactic acid bacteria and gluten-degrading enzymes to create more palatable products (11-13). Enzymatic degradation of gluten is also being investigated as an oral therapy in the form of a pill to be taken with gluten-containing food (14, 15). The intention is for this to be used to counteract intake of smaller amounts of gluten, typically ingested unintentionally. Targeting the permeability of the gut mucosa is an indirect way of alleviating symptoms from unintentional intake of small amounts of gluten (16). A possible way to cure celiac disease using a therapeutic vaccine is currently being investigated and a vaccine candidate has successfully undergone a Phase I study (17).

This thesis explored the possibility of blocking specific binding motifs for transglutaminase 2 that are present on gluten peptides. The idea of blocking the binding motifs is to prevent the gluten peptides from being identified and subsequently deamidated by transglutaminase 2, and thus impede their activation of the immune response. Similar approaches have been pursued previously by sequestering gluten (18) or by binding to gluten (19), but to my knowledge no previous study has specifically targeted the motifs involved in transglutaminase 2 binding to gluten. This thesis also investigated the effect of lactic acid fermentation on transglutaminase 2 interaction with gluten and the effect of extrusion cooking on gluten digestibility. Lactic acid fermentation can be used to degrade gluten, but incomplete degradation might be deleterious rather than protective. Digestion of gluten in the stomach and small intestine of humans has not been studied before but was done here in an ileostomy model. Extrusion cooking can change protein digestibility, and whether it also changes the digestibility of gluten peptides was examined after *in vivo* digestion in human ileostomy subjects.

## 2. OBJECTIVES

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The gluten-free diet is today the only treatment for celiac disease, but has several drawbacks. Thus, an important goal in research is to develop celiac-safe foods with improved quality. The overall aim of the work in this thesis was to investigate how the interaction between transglutaminase 2 and gluten peptides can be avoided. The main approach for preventing this interaction examined in the thesis was the possibility of blocking specific binding motifs for transglutaminase 2 on gluten peptides. The intention was to identify a molecule with the ability to bind to these specific sites on gluten, so that it could interfere with the binding of transglutaminase 2 and therefore stop the disease progression at an early stage. Lactic acid fermentation was investigated for its effect on gluten, with the focus on gluten peptides recognized by transglutaminase 2. Fermentation can degrade gluten, so this thesis investigated how the transglutaminase 2 interaction to gluten is affected by standard sourdough fermentation. It also examined how gluten protein is digested *in vivo*, by comparing digests obtained from ileostomy subjects after intake of an extruded gluten-containing product or a raw counterpart. Ileostomy contents provided a unique opportunity for studying digestion in humans and permitted investigation of how gluten is digested *in vivo*.

Specific objectives of the work presented in this thesis were to:

- Investigate how lactic acid fermentation affects transglutaminase 2 binding to gluten proteins in wheat flour and in baked breads (Paper I).
- Identify potential molecules with the ability to interact with transglutaminase 2 binding motifs in  $\alpha$ 2-gliadin, using computational models (Paper II).
- Assess the interaction between the identified molecules and gliadin, and the effect in preventing transglutaminase 2 enzymatic action on gliadin peptides *in vitro* (Paper II).
- Identify potentially harmful effects of a combination of one of the identified molecules, ascorbyl palmitate, and gliadin in cell cultures (Paper III).
- Investigate the digestion of gluten using digests from ileostomy subjects after ingestion of either a raw or an extruded gluten-containing product (Paper IV).

## 3. BACKGROUND

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### 3.1. Celiac disease

Celiac disease is a chronic autoimmune inflammatory enteropathy that may develop in genetically predisposed individuals, and is manifested upon ingestion of gluten or gluten-like proteins in wheat, barley, and rye. Celiac disease is by no means new, as the first known description of the disease was produced by Aretaeus of Cappadocia at some time between the 1<sup>st</sup> and 2<sup>nd</sup> centuries AD. The first 'modern' description was by Samuel Gee (1888), who suggested various diets for treatment, but failed to identify the exact components of the diet that caused the disease. The involvement of wheat, barley, and rye in celiac disease was first recognized by Wim Dicke in 1950 (for review, see Losowsky (20)).

#### 3.1.1. Symptoms and prevalence

Onset of celiac disease can occur at any age and it can be manifested in a multitude of ways, from severely symptomatic to more or less asymptomatic (2, 3). In the literature, there are many different ways of describing celiac disease. Ludvigsson et al. suggest the so-called Oslo definition for celiac disease and related terms, to bring consensus to the area (6). In classical celiac disease, there are symptoms of malabsorption combined with diarrhea, steatorrhea, weight loss, and/or growth failure (6). In non-classical celiac disease there is no malabsorption, but other gastrointestinal symptoms may be present (6). Gastrointestinal symptoms are more common in young children, but can be present in all age groups (21). Subclinical celiac disease is defined as being below clinical detection level, without strong enough signs or symptoms to suspect celiac disease (6). Some individuals have asymptomatic celiac disease, without any symptoms normally associated with celiac disease or that respond to a gluten-free diet. Individuals with subclinical and asymptomatic celiac disease are most often diagnosed in screening programs or identified in association with another primary diagnosis with a high risk of celiac disease (6). Most people with celiac disease have symptoms, which improve after starting on a gluten-free diet, but their symptoms are often not recognized (22, 23), leading to a long time before diagnosis and a large proportion of undiagnosed cases (22, 24-27). Extraintestinal symptoms include, but are not restricted to: anemia, dental enamel defects, dermatitis herpetiformis, arthralgia, fatigue, osteopenia/osteoporosis, amenorrhea, abnormal liver biochemistry, anxiety, depression, and irritability (21, 28). Celiac disease is more common in first-degree relatives (up to 20%), due to the genetic factor involved in the disease, but it is also a common comorbidity with autoimmune liver disease (up to 13%), type 1 diabetes (up to 12%), Down syndrome (up to 12%), Williams syndrome (up to 9%), IgA deficiency (up to 8%), autoimmune thyroid disease (up to 7%), and Turner syndrome (up to 5%) (28). Untreated celiac disease leads to reduced overall well-being, increased use of medication and healthcare services, and an increased risk of developing other autoimmune diseases and suffering from e.g., osteoporosis and infertility (21, 29). In terms of mortality, in individuals with undiagnosed celiac disease there are contradictory results ranging from no difference to nearly a four-fold increase in mortality (27, 30, 31).

In the general population in Europe or in countries where a large proportion of the population is of European descent, such as the US, the prevalence of celiac disease is approximately 1% (1). However, there are regional differences within these populations, e.g., the prevalence in Sweden and Finland is 2-3%, whereas in Germany it is only about 0.2% (1). The reasons for this have not been established. However, the higher consumption of dairy products in the northern countries may play a role, due to the high calcium content. Calcium is necessary for activation of the enzyme transglutaminase 2 (see section 3.3 of this thesis). The prevalence of celiac disease is also close to 1% in North Africa, the Middle East, and India (1),

while the highest recorded occurrence, 5.6%, has been found in the Saharawi population in Western Sahara (32). However, in these countries the diagnosis rate is generally low, as is awareness of the disease (1). The prevalence of celiac disease in China has previously been believed to be very low, but it has recently been found to be probably close to 1%, especially in the north of the country (33). Like most autoimmune diseases, celiac disease is more common in women and also often more symptomatic (34-36). The occurrence of celiac disease has increased several-fold in recent decades, possibly due partly to improved diagnostic practices and increased awareness, but also to an increase in the actual prevalence of the disease (2, 27, 37, 38). However, the reason for the increase has not been established.

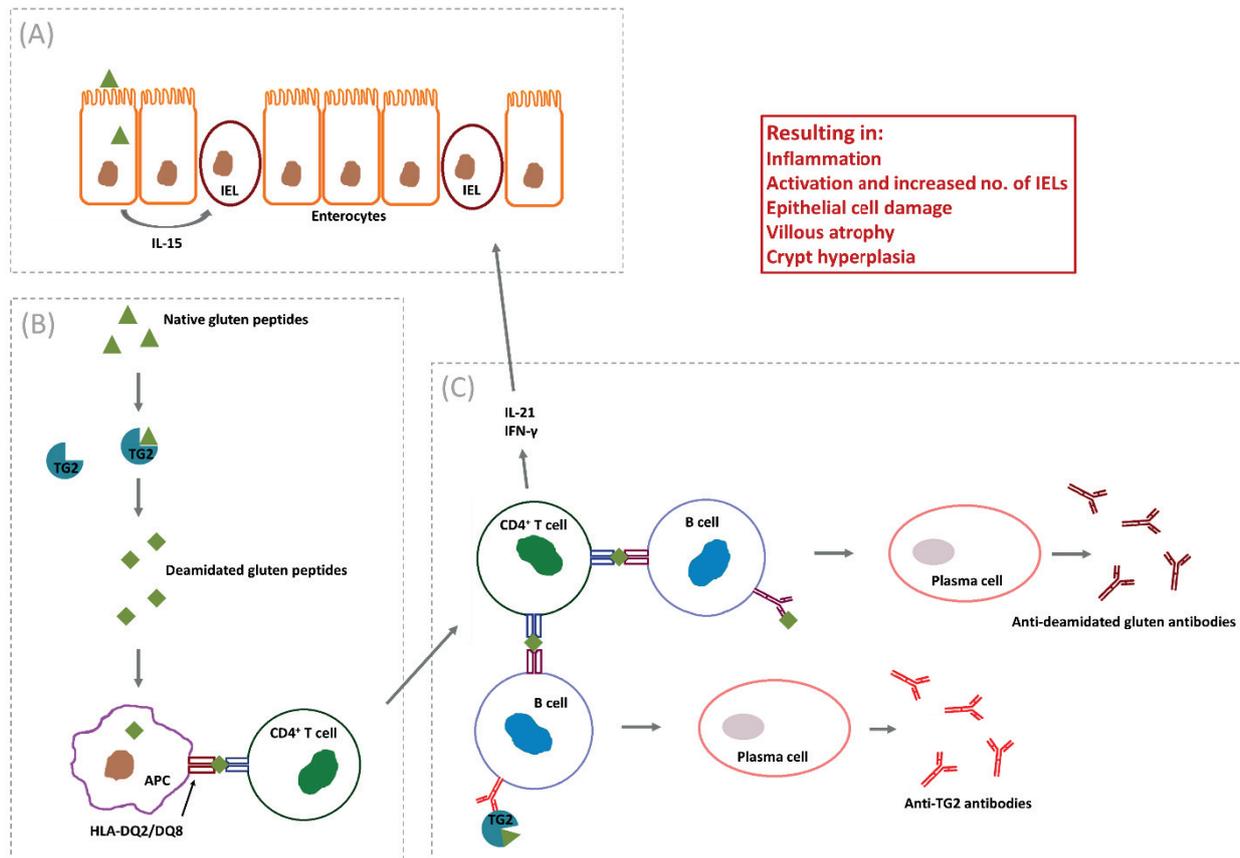
### 3.1.2. Genetic predisposition

Celiac disease may develop in genetically predisposed individuals. The main genetic factors are the human leukocyte antigen (HLA) class II molecules HLA-DQ2 and HLA-DQ8, which are expressed on antigen-presenting cells. A recent review reports that about 95% of celiacs have the HLA-DQ2 variant, while a minority have the HLA-DQ8 variant (4). A small proportion of celiacs have neither of the HLA-DQ2/DQ8 heterodimers, but often one half of the DQ2 heterodimer (39). However, in e.g., Europe, roughly 30-40% of the population have either of these two variants, but only a minority of these develop celiac disease (4, 40). So far, there are 43 known loci that have been shown to contribute to susceptibility for developing celiac disease, explaining approximately 50% of the heritability of this disease (4, 41). Celiacs do not necessarily carry all these risk loci, but different combinations of the loci affect the overall risk of developing celiac disease (4, 42). Many of the risk loci are also associated with other autoimmune diseases (4).

### 3.1.3. Immune response

The immune response in celiac disease involves both the innate and adaptive immune responses. In the adaptive part of the immune response, transglutaminase 2 (TG2) plays a crucial role. Gluten peptides and other dietary antigens that have survived gastrointestinal degradation are taken up by antigen-presenting cells and presented to CD4<sup>+</sup> T cells, which in a non-disease state would lead to oral tolerance to those antigens (43). In celiacs, this tolerance is lost for some reason, or has failed to develop, for certain gluten peptides. The gluten peptides are presented to CD4<sup>+</sup> T cells by antigen-presenting cells through HLA-DQ2/DQ8, but these have a relatively low affinity for native gluten peptides (44). However, transglutaminase 2-mediated deamidation of these gluten peptides greatly increases the affinity to the HLA molecules (5). The CD4<sup>+</sup> T-cells that recognize deamidated gluten peptides presented through HLA-DQ2/DQ8 differentiate into effector T cells that release pro-inflammatory cytokines such as IFN- $\gamma$  and IL-21 (4, 43). In turn, IL-21 and IFN- $\gamma$  activate intraepithelial lymphocytes (IELs), which are CD8<sup>+</sup> T cells embedded in the epithelial layer, leading to apoptosis of epithelial cells (4, 45). IL-21 also helps in activating B cells and induces them to differentiate into plasma cells, which secrete antibodies toward the deamidated gluten peptides (4, 43, 45). Plasma cells secreting autoantibodies directed against transglutaminase 2 are also present, but there are no CD4<sup>+</sup> T cells reactive against transglutaminase 2 that are able to promote B cell differentiation (43). Instead, transglutaminase 2 is likely to contribute to its autoantibodies by creating cross-links between itself and gluten peptides, either by the intermediate thioester formation preceding deamidation/transamidation or by forming isopeptide bonds with lysine residues present in the enzyme (46, 47). Certain gliadin peptides also have the ability to induce an innate immune response by triggering intestinal epithelial cells to express IL-15, leading to IEL activation and epithelial cell damage (48, 49). Moreover, certain gliadin peptides are able to alter epithelial barrier function and thereby increase barrier permeability (50, 51). There are many immunogenic gluten peptides,

but the response to these in celiacs is heterogeneous, so peptides eliciting a response in one celiac may not do so in all others (52). Overall, the immune response leads to inflammation and activation and increased numbers of IELs, resulting in epithelial cell damage, villous atrophy, and crypt hyperplasia, which are characteristic of celiac disease. The complete response is complex and involves many other aspects of the immune system not mentioned here. See Figure 1 for a summary of the celiac immune response.



*Figure 1. Immune response in celiac disease. (A) Certain gluten peptides are able to induce an innate immune response in intestinal epithelial cells by release of IL-15, leading to intraepithelial lymphocyte (IEL) activation, epithelial cell damage, and increased permeability. (B) Native gluten peptides are deamidated by transglutaminase 2 (TG2) and taken up by antigen-presenting cells (APCs), such as dendritic cells, and presented through HLA-DQ2/DQ8 to CD4+ T cells. (C) Activated gluten-specific CD4+ T cells release IL-21 and IFN- $\gamma$ , further activating IELs and leading to epithelial apoptosis. These CD4+ T cells also activate B cells presenting deamidated gluten peptides through HLA-DQ2/DQ8, inducing them to differentiate into antibody-secreting plasma cells. B cells recognizing deamidated gluten peptides produce antibodies to these deamidated gluten peptides. However, instead of presenting TG2-derived peptides on their HLAs, B cells recognizing TG2 may present deamidated gluten peptides that are either cross-linked to TG2 or in the thioester intermediate, thus leading to autoantibodies to TG2. The results characteristic for celiac disease are inflammation, and activation and increased numbers of IELs, resulting in epithelial cell damage, villous atrophy, and crypt hyperplasia.*

Diagnosis of celiac disease is based on a combination of histological analysis of duodenal biopsies, various serological tests, and genetic testing for HLA-DQ2/DQ8 (28, 53). For histological and serological tests, it is necessary for the individual to be on a gluten-containing diet to ensure correct diagnosis. Human leukocyte antigen testing can be performed to rule out celiac disease at an early stage, e.g., for people already on a gluten-free diet, or when facing contradictory test results. The main serological test is IgA anti-TG2, measured together with total serum IgA (to avoid a false negative result in IgA-deficient individuals) (28). Other useful serological tests include IgG anti-TG2, IgG anti-deamidated gliadin, and IgG endomysial

antibodies (EMA) (28, 53). If serological tests are positive, or negative but with strong indications of celiac disease, histological analysis of duodenal biopsies can be used to confirm diagnosis. At least five biopsies should be taken, since the mucosal damage is irregular in many cases (53). Typical findings are blunted or atrophic villi, crypt hyperplasia, and increased numbers of intraepithelial lymphocytes (53).

#### 3.1.4. Wheat allergy and non-celiac gluten sensitivity

Apart from celiac disease, which is the focus in this thesis, people can also be allergic to wheat proteins or suffer from the poorly defined disorder called non-celiac gluten sensitivity. Wheat allergy, like other allergies, is characterized by T helper type 2 inflammation, whereas celiac disease, like other autoimmune diseases, is characterized by T helper type 1 inflammation (54). Wheat allergy can be mediated by IgE antibodies or by an inflammation with eosinophils and T cells, and can be triggered by either ingesting or inhaling allergens (54). Many different proteins in wheat are allergenic, including several gluten proteins (55). Non-celiac gluten sensitivity is not well characterized and somewhat controversial. Gluten has been accused of triggering intestinal and extraintestinal symptoms in individuals without celiac disease or wheat allergy. The symptoms are similar to those of celiac disease and irritable bowel syndrome (IBS) (56). However, in a double-blind cross-over trial with 37 subjects with non-celiac gluten sensitivity, no negative effects were found for gluten. Instead, it appears that fermentable oligo-, di-, monosaccharides and polyols (the so-called FODMAPS), which are poorly absorbed but fermentable short-chain carbohydrates, might be the cause (57). Further investigations are needed to establish the pathogenesis of this disorder.

### 3.2. Gluten protein

Wheat is one of the most important crops worldwide, with production of approximately 750 million tons in 2016 (58). The vast majority of the wheat grown, roughly 95%, is hexaploid bread wheat and the second most common type is tetraploid durum wheat (59). While bread wheat is by far the most common, there are many thousand different wheat varieties with different properties and growth conditions. The protein content of wheat typically ranges between 8-15% (59), and of this around 80% is gluten proteins (60). Gluten has traditionally been defined as the rubbery mass that is left after starch and other water-soluble constituents have been washed away from a wheat dough. This mass consists of around 80% proteins and 5-10% lipids, and the rest is a mixture of starch and other carbohydrates (61). However, in general and throughout the remainder of this thesis, the term gluten is used to refer only to the proteins. Barley and rye contain similar proteins to wheat gluten, called hordein and secalin respectively, but when referring to 'gluten' all three are often combined, since they have all been shown to elicit immune responses in celiac disease. Oats also contain proteins similar to gluten, called avenin, but these proteins are not as closely related to gluten as secalin and hordein, and are less abundant (62). It has been debated whether oats are safe for celiacs or not, either by being immunostimulatory themselves or by contamination with gluten, but it appears that the vast majority of celiacs can tolerate oats if they are free of contaminating gluten (62, 63).

Gluten proteins are characterized by a high content of glutamine (approximately 35 mol%), proline (15 mol%), and hydrophobic amino acids (19 mol%), and can be divided into two main groups: the gliadins and the glutenins (61), see Table 1. In the seeds, the function of gluten is as a storage protein. Wheat gluten, and to a smaller extent rye secalin, have a special and very desirable property in that they impart viscoelasticity to the dough, allowing carbon dioxide to be trapped during leavening of bread (59). Both the gliadins and the glutenins contribute to the viscoelasticity, but in different ways. The gliadins, which are mainly monomers, are mostly responsible for the viscosity and extensibility of the dough, whereas the

glutenins, which can form large aggregates, are cohesive and elastic, which gives the dough both strength and elasticity (64). Gliadins can be divided into over 100 different components that are combined into three main groups ( $\alpha$ ,  $\gamma$ , and  $\omega$ ) based on their mobility during gel electrophoresis at low pH (64). The  $\alpha$ -gliadins were previously classified as two separate groups ( $\alpha$  and  $\beta$ ), but are now combined based on amino acid sequence similarity, while the  $\omega$ -gliadins can be further divided into  $\omega$ 5- and  $\omega$ 1,2-gliadins (64). The  $\alpha$ - and  $\gamma$ -gliadins make up roughly 30% each of the gluten fraction, whereas the  $\omega$ -gliadins only comprise around 10%. The molecular weight typically ranges from 28 to 35 kDa for  $\alpha/\beta$ -gliadins, 31-35 kDa for  $\gamma$ -gliadins, and 39-55 kDa for  $\omega$ -gliadins (64). Glutenins form aggregates linked by interchain disulfide bridges, resulting in proteins with molecular weight from 500 kDa to over 10 000 kDa, making them one of the largest natural proteins (64). By breaking the disulfide bonds, the glutenins can be divided into two main subunit groups: low molecular weight glutenin subunits (LMW-GS) and high molecular weight glutenin subunits (HMW-GS). The LMW-GS are the main type, comprising roughly 20% of the gluten fraction, and they are similar in both amino acid composition and size to the  $\alpha$ - and  $\gamma$ -gliadins (32-39 kDa) (64). The HMW-GS are larger (67-88 kDa) and less abundant, making up approximately 10% of the gluten fraction (64).

*Table 1. Molecular weights and proportions of the total gluten content for the different gluten fractions. Adapted from Wieser (64).*

Gluten fraction	MW (kDa)	Proportion of total gluten (%)
<i>Gliadins:</i>		
$\alpha$ -gliadin	28-35	28-33
$\gamma$ -gliadin	31-35	23-31
$\omega$ 1,2-gliadin	39-44	4-7
$\omega$ 5-gliadin	49-55	3-6
<i>Glutenins:</i>		
LMW-GS	32-39	19-25
HMW-GS	67-88	7-13

A very important aspect of gluten proteins with regard to celiac disease is that their high content of proline makes them highly resistant to gastrointestinal degradation in humans (65-67). This, together with the high glutamine content, makes them excellent substrates for transglutaminase 2, which deamidates the gluten peptides, leaving them highly charged, and thus promotes the binding to HLA-DQ2/DQ8 proteins on antigen-presenting cells, starting a celiac immune response.

### 3.3. Transglutaminase 2

The enzyme transglutaminase 2 (TG2), also referred to as tissue transglutaminase (EC 2.3.2.13), is a member of the widely distributed transglutaminase family (68). The transglutaminases are encoded by closely related genes with a high degree of sequence similarity and catalytic mechanism, but they have different physiological functions and expression in different tissues and during different developmental stages (69). Transglutaminase 2 is expressed ubiquitously and is localized to the cytosol, nucleus, mitochondria, cell membrane, and extracellular space, as reviewed by Park et al. (70). Besides its transglutaminase activity, transglutaminase 2 has several other functions, e.g., as a G protein, protein

disulfide isomerase, protein kinase, and DNA nuclease, as well as a role in cell-extracellular matrix interactions through direct interaction with e.g., fibronectin and integrin (70). Transglutaminase 2 plays a role in numerous cellular processes, such as apoptosis, wound healing, migration, differentiation, and inflammation (70).

The transglutaminase activity is dependent on calcium ions ( $\text{Ca}^{2+}$ ) in the millimolar range, leading to exposure of the active site to the substrate (71). In contrast, guanosine triphosphate (GTP) and guanosine diphosphate (GDP) inhibit its catalytic activity by stabilizing the closed conformation (72). In addition, the zinc ion ( $\text{Zn}^{2+}$ ) inhibits transglutaminase 2 activity by competing with  $\text{Ca}^{2+}$  for binding sites (73). In its active form, the enzyme links the  $\gamma$ -carboxamide of a glutamine residue with a cysteine residue in the active site through an acylation reaction, thus forming a  $\gamma$ -glutamylthioester bond which releases ammonia (69, 74). The second part of the reaction can take one of two directions, either a transamidation or a deamidation reaction. In the transamidation reaction, a primary amine, such as peptide-bound lysine, attacks the thioester bond and creates an intermolecular isopeptide  $\epsilon$ -( $\gamma$ -glutamyl)lysine cross-link through a deacylation reaction (69, 74). In the deamidation reaction, water is used instead of a primary amine, leading to deamidation of glutamine into glutamic acid through a hydrolysis reaction. After this, the active center of transglutaminase 2 is restored and is thereby ready to take part in another round of catalysis (69, 74). At  $\text{pH} \geq 7$  and in the presence of primary amines, the transamidation reaction is favored over the deamidation reaction but deamidation is not silenced, e.g., at  $\text{pH} 7.3$  and with an excess of primary amine, some deamidation still occur (75). At  $\text{pH} 5.5$ - $6.5$  the deamidation reaction is favored, but proceeds at a lower reaction rate than at a higher  $\text{pH}$  (75). In the transamidation reaction, the formation of a thioester intermediate is the rate-limiting step (74), while in the deamidation reaction, hydrolysis is the rate-limiting step (76). Under normal physiological conditions, it is believed that most of the extracellular transglutaminase 2 is in its closed formation, but that e.g., tissue injury is able to transiently activate extracellular transglutaminase 2, as well as releasing intracellular transglutaminase 2 (77). Transglutaminase 2 expression, and transglutaminase activity, are increased in the intestinal mucosa in people with active celiac disease (78, 79). The increased expression has been attributed to the inflammatory response of celiac disease, in particular  $\text{IFN-}\gamma$  (78).

Transglutaminase 2 substrate specificity has been investigated in several studies, with slightly varying results. There is consensus that the key sequence for transglutaminase 2 binding is QXP (where X denotes any amino acid) and that sequences containing QP and QXXP are not targeted (52, 74, 75). The surrounding amino acids most likely also contribute to binding specificity, but to a lesser extent. It has been found that the residue in position -1 to +3 from the targeted glutamine influences specificity (75). Several amino acids are able to increase binding specificity slightly at position -1 (FYWSAGQNTVPD), at position +1 (RKHYWFILVQASTED), at position +2 (P strongly, WYFVALMIQH slightly), and at position +3 (ILVfyWTS) (75). It has been shown that the sequences QXXF(Y, W, M, L, I, or V) are also targeted (52), or that QXY may be targeted and that a hydrophobic residue is favored in the +3 position, either as QXPh or QXXh (where h denotes a hydrophobic amino acid) (52, 74). Glutamine and proline are the most abundant amino acids in gluten and QXPfY is a frequently occurring sequence, thus making gluten an attractive substrate for transglutaminase 2.

Transglutaminase 2-mediated deamidation of gluten peptides has been suggested to take place in several different tissues where extracellular transglutaminase 2 is present, including in the sub-epithelial region, even with primary amines present (75). Deamidation can also occur in the brush border, where the  $\text{pH}$  is slightly more acidic than in the sub-epithelial region, thus favoring deamidation, or it can occur in

endosomes when transglutaminase 2 and gluten have been endocytosed (75). It has also been suggested that deamidation can occur in lymphoid tissues, as it is known that transglutaminase 2 is expressed in these tissues too (80).

Autoantibodies to transglutaminase 2 have two proposed origins. First, transglutaminase 2 contains lysine and can use some of these residues as a secondary substrate in the deacylation reaction and cross-link the primary substrate, such as gluten peptides, to itself (46, 81). Certain gluten peptides can thus be deamidated at one site and at the same time transamidated onto transglutaminase 2 at another site. Second, autoantibodies may also be created from uptake by antigen-presenting cells of the thioester intermediate, possibly with previous deamidation at another site in the same peptide (82, 83).

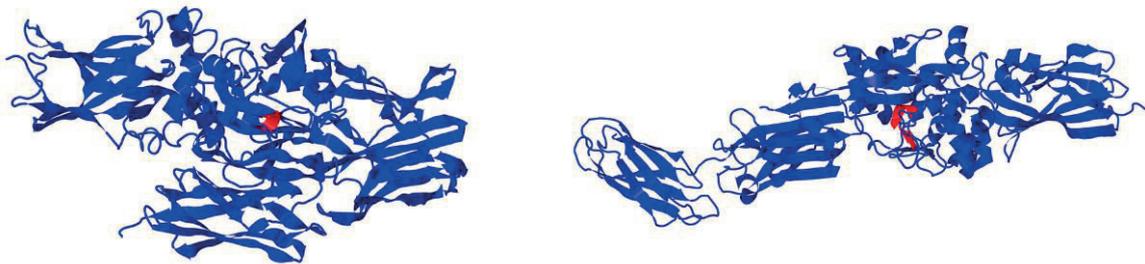


Figure 2. Inactive (closed) conformation and active (open) conformation of transglutaminase 2, with the catalytic site in red. Illustration prepared in Jmol from pdb-files 4PYG and 2Q3Z obtained from the Protein Data Bank.

### 3.4. Risk factors for celiac disease development

#### 3.4.1. Modern wheat varieties and increased gluten intake

It has been hypothesized that selective breeding during the past 10,000 years has resulted in wheat varieties with a higher gluten content, and that modern varieties high in gluten are part of the explanation for the increase in celiac disease (84). However, this does not seem to be the case. The protein and gluten content in modern varieties of wheat in the USA have not changed considerably during the past 100 years (85) and in the UK there has been a clear decline in wheat protein content over the past 150 years or so (86). In a study which estimated the gluten content in 245 accessions from the *Triticeae* tribe, including both domesticated and wild wheat, barley, and rye, it was found that the gluten content was lower in the domesticated varieties (87). Thus, breeding seems to have focused more on yield, resulting in more starch, than on protein content (86, 87). However, *Triticum aestivum*, commonly called bread wheat, which is the most widely cultivated wheat species, has a higher proportion of known celiac disease immunogenic epitopes than other wheat types (87). It is claimed in one study that that the modern varieties of wheat have a higher content of the gli $\alpha$ -9 epitope, known to be highly immunogenic (88). However, another study including more than just one known immunogenic epitope found that modern varieties actually contain less immunogenic epitopes (89). Similarly, tests on various wheats, including several ancient strains, for their immunogenicity in gluten-specific T cells taken from celiacs indicate that all are immunogenic (90).

Another consideration is that the per capita consumption of wheat has not changed considerably in industrial countries during the past 50 years (only 6% increase from 1963 to 2003) (91). In Europe, the overall mean caloric intake originating from cereals has actually decreased during this period (91). In the USA, the average intake increased by approximately 20% from 1970 to 2008, but before 1950 the average intake was substantially higher (85).

One aspect of gluten consumption that is likely to have increased is intake of the so-called 'vital gluten' or 'wheat vital gluten'. Basically, vital gluten is produced by washing away the starch from wheat and then carefully drying it, thereby retaining the beneficial viscoelastic properties at rehydration (92). Vital gluten is today an important ingredient in foods, where its primary use is to increase the gluten content in baking and in flour production, but it is also used as an ingredient in processed meats and breakfast cereals such as Kellogg's Special K (92). According to recent estimates, the consumption of vital gluten in the USA has tripled since 1977, but the amount of vital gluten is still less than 10% of the gluten consumed in wheat (85). Although, as yet no study has evaluated the role of vital gluten in celiac disease, or whether it provokes an immune response to the same extent as naturally occurring gluten.

#### 3.4.2. Effects of microbiota

The gut microbiota has been associated with celiac disease in several studies, as reviewed by Cenit et al. (93). Intestinal dysbiosis has been observed in people with untreated celiac disease and those on a gluten-free diet, compared with healthy controls. However, it is not yet certain what is cause and what is effect; e.g., whether the dysbiosis leads to or increases the risk of celiac disease, or whether ongoing celiac disease alters the microbiota, leading to dysbiosis. Cenit et al. argue that the dysbiosis probably acts by exacerbating the progression and symptoms of celiac disease, but that it is also likely to contribute to onset of celiac disease (93). Increased progression of celiac disease has been demonstrated in germ-free mice colonized with bacteria taken from celiacs or healthy controls (94). The bacteria used in that study were selected based on their capability for degrading gluten. The results showed that bacteria from celiacs produced degraded gluten peptides that were better at entering the mouse intestinal barrier and were more efficient in activating gluten-specific T-cells taken from celiacs (94). The role of gut microbiota in celiac disease could be partly genetic, as some of the identified genes associated with celiac disease are related to bacterial colonization and sensing (93). Diet and other environmental factors also naturally influence the composition of the microbiota. Use of antibiotics (95) and proton pump inhibitors (96) has been associated with an increased risk of subsequent development of celiac disease, but there is a possibility that undiagnosed celiac disease led to prescription of these drugs and that they are not a contributing factor *per se*.

Several types of infections have been associated with an increased risk of celiac disease development, e.g., frequent rotavirus infections have been shown to increase the risk of celiac disease (97). In a cohort of almost 2000 children with HLA-DQ2/DQ8, monitored for rotavirus antibodies and autoantibodies for transglutaminase 2 from 9 months of age, the risk of developing celiac disease was increased by over three-fold for  $\geq 2$  rotavirus infections (97). Infections with campylobacter (98), reovirus (99), and hepatitis C (100) have also been associated with an increased risk of developing celiac disease. One theory for the increase not just in celiac disease, but in all types of autoimmune diseases and allergies, in developed countries is the 'hygiene hypothesis'. This hypothesis is described in e.g., reviews by Sironi & Clerici (101) and Kondrashova et al. (102). The main idea is that, since humans have evolved in the constant presence of various microorganisms such as bacteria and parasites, these are involved in the development and regulation of the human immune system (101, 102). In developed countries, humans today are less exposed to many of these microorganisms, especially parasites such as helminths. As a consequence, according to the hygiene hypothesis, the immune system is not sufficiently stimulated, leading to an increased risk of developing autoimmune diseases and allergies. Kondrashova et al. compared the incidence of autoimmune diseases and allergies in Finland with that in the bordering region of Karelia, Russia. These two populations have a fairly similar genetic background, but there are large differences in

several environmental and lifestyle-associated factors, e.g., the incidence of several types of infections is much higher in Russian Karelia and gross national product is roughly 15-fold higher in Finland (2001). The results showed that all the autoimmune diseases and allergies investigated were several-fold more common in Finland than in Russian Karelia, with celiac disease being more than four times as common (102). Thus, both the presence and the absence of certain microorganisms might affect the risk of developing celiac disease.

#### 3.4.3. Other possible risk factors

It has been suggested that the use of microbial transglutaminases in the food industry might be a contributing factor to the rise in celiac disease (103). Microbial transglutaminases are used to improve e.g., certain meat and fish products, milk and dairy products, and commercially baked goods, and have become increasingly popular. A common Western diet might contain up to 15 mg of microbial transglutaminase. As is the case for human transglutaminase 2, gluten is a preferred substrate of microbial transglutaminase, and it has been shown that gluten treated with microbial transglutaminase is able to trigger a celiac disease-relevant immune response. Gluten can also be chemically deamidated, making it more easily dispersible, and used in foods for emulsification and for stabilizing foam (92). Thus, deamidation of gluten peptides may occur already before ingestion, thereby increasing the risk of developing celiac disease (103). However, this has yet to be verified in scientific studies.

Sweden experienced a four-fold increase in celiac disease incidence in children starting in 1985 and lasting until 1995, when incidence “normalized” (104). At the time, possible reasons for the increased incidence were believed to be a combination of increased amounts of gluten in weaning foods and reduced breastfeeding. The European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), which provides recommendations for gluten introduction in infants, recommended introduction between 4-7 months of age, while still breastfeeding. However, as Lionetti et al. highlight in their review, age of introduction and breastfeeding have both been shown not to significantly affect the risk of celiac disease development (105). However, earlier introduction is associated with earlier development of celiac disease (106). The current ESPGHAN recommendation is to introduce gluten between 4 and 12 months of age, and to avoid consumption of large amounts during infancy (106).

### 3.5. Theories on the initiation of celiac disease and the role of zinc

Several risk factors for developing celiac disease are known or suggested, but it is still not known exactly what leads to tolerance to gluten failing to establish, or being lost, in only a minority of people presenting with these factors. Much is known about the pathophysiology of celiac disease once tolerance is broken, but not about the triggering factors. Koning proposes a so-called “multiple hit model” in which activation of the innate immune system in a genetically pre-disposed individual, caused by e.g., an infection, could lead to polarization of the immune response that may spread and ultimately lead to disease (107). However, it is not known what specific innate events are necessary to initiate the polarization and T-cell response to gluten (107). Stenberg et al. earlier proposed a similar hypothesis for initiation, but with more focus on the role of zinc (76). In their hypothesis, the innate immune system is triggered by e.g., stress, infection, or inflammation, which redistributes zinc, leading to reduced zinc level in the intestinal wall. Transglutaminase 2 present in the intestine, or from macrophages attracted to the site, is activated due to the reduced zinc level and may deamidate certain gluten peptides, resulting in the more well-known activation of T cells, production of antibodies toward deamidated gliadin and transglutaminase 2,

increased inflammation, and ultimately villous atrophy. The sequential malabsorption reduces zinc uptake, which together with the ongoing inflammation further propagates the disease (76).

Zinc is an essential micronutrient and is the second most abundant trace element in human cells, giving a total of approximately 2-3 g in the body (76, 108). There are no substantial stores of zinc in the body, so homeostasis of zinc is maintained mainly in the gastrointestinal tract and is affected by food intake and intestinal absorption, together with losses in urine and feces (76, 108). Zinc in the body is predominantly in the form of  $Zn^{2+}$  and is reversibly bound to proteins (76, 109). Zinc is required by roughly 300 enzymes for full activation, many of which are related to the immune system (76). The importance of zinc for the immune system has been reviewed elsewhere, e.g., by Shankar & Prasad (109). It is known that individuals suffering from zinc deficiency have suppressed immunity, with e.g., both lower count and activity of lymphocytes, and an increased risk and severity of infections, as well as damage to the epithelia of the gastrointestinal and pulmonary tracts (109). Zinc deficiency is a problem in some developing countries, especially in conjunction with persistent diarrhea (110, 111). It is also well known that zinc deficiency is common in persons with untreated celiac disease (112, 113). However, it has not been established whether the low zinc levels are only a consequence of celiac disease, or also promote initiation of celiac disease.

One difference between old and modern wheat varieties is micronutrient density, with modern wheats having significantly lower content of e.g., iron and zinc (86). The reduced content of these micronutrients is mostly attributed to yield dilution, i.e., since the increase in yield is mostly due to an increase in starch, the other nutrients are reduced in density (86). The gluten content in modern wheat is probably not to blame for the increase in celiac disease prevalence, but the reduced content of zinc may play a role, as zinc is known to be a potent inhibitor of transglutaminase 2.

## 3.6. Treatment of celiac disease

### 3.6.1. The gluten-free diet

A gluten-free diet is to date the only available treatment for celiac disease. A gluten-free diet excludes gluten from wheat, barley, and rye. Pure oats are likely to be safe for almost all celiacs, but are often contaminated with gluten from processing facilities handling several kinds of grains. If followed strictly, a gluten-free diet will efficiently relieve symptoms in a vast majority of individuals. Gluten-free products have gained popularity in recent years, partly due to increased awareness and prevalence of celiac disease, but also among people without a medical need to avoid gluten, resulting in substantial growth in the gluten-free food segment (114).

However, there are several significant drawbacks with a gluten-free diet. Despite increasing popularity, gluten-free products are still less available and more expensive than their gluten-containing counterparts (7, 8, 115). Moreover, although the overall quality has improved in recent years, many gluten-free products, especially breads, are still often considered to have inferior sensory quality (116). Maintaining a gluten-free diet can be difficult, partly due to the cost and availability, but also because gluten is commonly used as an ingredient in a variety of products (12, 92). Compliance with a gluten-free diet is poor in adults with celiac disease, e.g., several studies have found that less than 50% manage to adhere to a strict long-term gluten-free diet (9, 10, 117). There are also concerns regarding the nutritional content of a gluten-free diet. Many gluten-free products have a high caloric content, originating from fat and carbohydrates, and a gluten-free diet has been seen to increase the risk of overweight and obesity, as reviewed by Gobbetti (12). In one study, two out of 98 persons diagnosed with celiac disease fulfilled the criteria for

metabolic syndrome at diagnosis, whereas after one year on a gluten-free diet that figure increased to 29 out of 98 (118). Furthermore, celiacs on a gluten-free diet show an increased risk of having suboptimal intake of several minerals, vitamins, and dietary fiber (12). It is of course possible to achieve a satisfactory nutritional intake while on a gluten-free diet, but this requires knowledge and determination in the individual. The overall quality of gluten-free products can be increased by using carefully selected gluten-free raw materials with high nutritional value, additives, and sourdough fermentation, but it is difficult to fully replace gluten (116).

Foods labeled gluten-free can contain up to 20 mg gluten/kg product, according to the Codex Alimentarius Commission, which is part of the Joint FAO/WHO Food Standards Programme (119). This level has also been adopted by the European Union and the USA. The actual tolerable amount of gluten that can be consumed without any measurable negative effect for a celiac is reported to be difficult to assess and varies between individuals, but should generally be kept below 50 mg/day (120). However, certain individuals may react to as little as 10 mg/day (120).

### 3.6.2. Novel therapies

Several interesting novel therapies for celiac disease are currently being investigated, but so far none has progressed further than Phase II trials. One approach is to degrade gluten in the gut by taking a pill containing enzymes capable of degrading gluten. This could be compared to pills degrading lactose used by lactose-intolerant people. However, the consequences of failure, e.g., if the enzyme fails to degrade enough gluten due to too much being present or if the food matrix in some way hinders degradation, are much more severe for someone with celiac disease compared with someone with lactose intolerance. Two formulations of gut-degrading gluten enzymes have so far reached clinical trials: ALV003 (15) and AN-PEP (14). Both of these have shown promising results, but neither is intended to enable a normal gluten-containing diet, but rather to help protect the individual from smaller amounts of unintentional intake of gluten while on a gluten-free diet. A complementary approach is to modulate the permeability of the gut mucosa. The inflammatory response in celiac disease increases the paracellular permeability in the epithelial layer by opening tight junctions, enabling more gluten to enter. Larazotide acetate (AT-1001) is able to inhibit tight junction opening and in a Phase II trial has successfully relieved symptoms in celiacs with persistent symptoms despite being on a gluten-free diet (16). Larazotide acetate is thus not intended to replace a gluten-free diet. Another approach is to induce immune tolerance to gluten through a therapeutic vaccine, e.g., Nexvax2 has undergone Phase I studies with positive results (17). In these studies, the vaccine contained three peptides known to engage gluten-specific CD4<sup>+</sup> T cells and was administered intradermally twice a week over a period of eight weeks in subjects with celiac disease. The idea is to make the CD4<sup>+</sup> T cells unresponsive to the administered peptides and thereby hopefully to cure celiac disease. The vaccine seems to be well tolerated, but further studies are needed to establish its efficiency. Other approaches to alleviate celiac disease that have undergone clinical trials include sequestering gliadin using the so-called BL-7010 copolymer (18), hookworm infection to promote gluten tolerance (121), and the use of a *Bifidobacterium* as a probiotic (122). As reviewed by Plugis & Khosla, pre-clinical trials include, but are not restricted to, inhibiting transglutaminase 2, blocking HLA-DQ2/DQ8, and blocking IL-15 (123).

Fermentation with lactic acid bacteria as a way to degrade gluten protein has been reported in several studies (124-128). The rationale for degrading gluten before e.g., baking a bread is that the end product will have higher perceived quality compared with most naturally gluten-free breads, while at the same time being rendered safe for celiacs. However, by degrading the gluten proteins, some of desired

properties of wheat are also inevitably destroyed, since gluten is responsible for e.g., the viscoelastic properties. It will therefore be difficult, if not impossible, to achieve the same level of perceived quality with fully degraded gluten. Another issue is that incomplete degradation of gluten, instead of being beneficial for celiacs, may actually be negative in that it creates more peptides with the potential to be deamidated that can more easily enter the intestinal cells. A small study has found that the oral microbiome of celiacs is significantly different from that of healthy controls, with higher gluten-degrading activities in the celiacs (129). However, the contribution of oral microbiota in degrading gluten *in vivo* is still unknown.

Blocking gliadin from recognition by transglutaminase 2 has previously been investigated using synthetic peptides (19, 130). However, the selection of peptides using a phage display method in these studies was not specifically targeted against transglutaminase 2 binding motifs on gliadin. The transglutaminase 2 transamidation of gliadin was found to be reduced by roughly 30% using these peptides (19). Even though this is an interesting approach, a 30% reduction is too little to be a viable method for making celiac-safe products.



question. Throughout this thesis, the term *in vitro* digestion refers to simulation of the gastric and proximal duodenal digestion phases. The rationale for ending the digestion at the beginning of the small intestine is that the first half of the duodenum (including transglutaminase 2) is already exposed to gluten peptides before the secretion of pancreatic enzymes. The major component of the oral phase is  $\alpha$ -amylase, which degrades starch, and was omitted here since only pure protein (gliadin) was digested in the model. The digestion model did not contain any microorganisms.

## 4.2. Lactic acid fermentation

In Paper I, the aim was to investigate how lactic acid fermentation of wheat flour, resembling a typical sourdough fermentation, affects the interaction of transglutaminase 2 with gluten. Two methods were used to assess transglutaminase 2 binding potential, measuring either the amount of  $\alpha$ 2-gliadin or the level of transamidation, both in pure wheat flour and as part of a sourdough bread.

### 4.2.1. Fermentation of wheat flour and sourdough breads

Three different species of *Lactobacillus* were used: *L. brevis*, *L. plantarum*, and *L. pentosus*. These were used for fermenting wheat flour (as a slurry) and for preparing sourdough breads. These three species were chosen because they are all commonly found in sourdough cultures. It is important to note that the strains were not chosen based on gluten-degrading capacity and that each species was used by itself. After fermentation, the samples were subjected to an *in vitro* digestion (see description in section 4.1 of this thesis). The fermentation of the wheat flour (5 g wheat flour in 70 mL water) was carried out at 37 °C for 21 hours. The sourdough comprised water and wheat flour in equal amounts (w/w) and was fermented for 24 hours at room temperature. The final bread contained 20% sourdough and commercial baker's yeast was added and allowed to act for a total of 4 hours during the baking process.

### 4.2.2. $\alpha$ 2-gliadin in fermented wheat flour and sourdough breads

The amount of  $\alpha$ 2-gliadin was measured in both the fermented wheat flour and the sourdough breads using a commercial ELISA kit (GlutenTox ELISA Competitive; Biomedal Diagnostics, Seville, Spain), which is based on an antibody specific for the amino acid sequence QPQLPY conjugated with horseradish peroxidase (HRP) for detection (G12-HRP). The  $\alpha$ 2-gliadin peptide is considered one of the most immunogenic peptides for celiacs (66) and the detected amino acid sequence, which is repeated three times in  $\alpha$ 2-gliadin, contains a binding site for transglutaminase 2 (QLP). If the fermentation were to degrade gluten successfully, the amount of this peptide would decrease.

### 4.2.3. Transamidation of fermented wheat flour

The transamidation activity was measured in the fermented wheat flours using a method based on the work of Skovbjerg et al. (131). In brief, the samples are coated on a 96-well plate and a recombinant human transglutaminase 2 (T022; Zedira GmbH, Darmstadt, Germany) transamidates the substrate 5-(biotinamido)pentylamine (Thermo Fisher Scientific, Waltham, MA, USA) to plate-bound gluten peptides. Streptavidin labeled with europium (Perkin Elmer, Waltham, MA, USA), which has a high affinity for the biotin part of the substrate, is then added. Finally, the fluorescence of the europium can be measured (345 nm excitation, 617 nm emission). If the fermentation were to degrade gluten successfully, the level of transamidation would decrease. This method does not consider any deamidation reactions that might occur, but with access to a primary amine as a substrate the transamidation reaction is strongly preferred by transglutaminase 2.

### 4.3. Preventing transglutaminase 2-gluten interactions through binding molecules

In Paper II, the aim was to identify potential food additives with the ability to bind to transglutaminase 2 binding motifs on gluten peptides, thereby blocking transglutaminase 2 from binding to these motifs. The work in Paper II consisted of two parts: 1) computer modeling of  $\alpha$ 2-gliadin and virtual screening of molecules docking to  $\alpha$ 2-gliadin (which was performed by Patricia Saenz-Méndez) and 2) *in vitro* assessment of the most promising molecules identified in the screening process. In Paper III, the most promising molecule identified, ascorbyl palmitate, was studied in Caco-2 cell cultures to assess its safety in combination with gliadin.

#### 4.3.1. Computer modeling of $\alpha$ 2-gliadin and virtual screening of docking molecules

In brief, a homology model of  $\alpha$ 2-gliadin was built with YASARA software using the crystal structures of five templates obtained from RSBC Protein Data Bank (PDB id: 1S9V8, 4OZF, 4OZG, 4OZH, 4OZI9). The  $\alpha$ 2-gliadin peptide was chosen for modeling for two main reasons. First, it is considered to be the most immunogenic peptide in celiac disease that withstands gastrointestinal degradation, and second, its size (33 amino acids). Initially, attempts were made to construct a model of whole  $\alpha$ -gliadin, but it was too large to be feasible (290 amino acids). Models were built by using alternative alignments for each template through a stochastic approach, side-chains were built in, and a combination of steepest descent and simulated annealing minimization was performed. The entire model was then subjected to simulated annealing minimization and the final model was evaluated through the PROCHECK analysis, by calculating the percentage of conformations in favored regions obtained from Ramachandran plots.

The homology model of  $\alpha$ 2-gliadin was then used as a basis for virtual screening studies for identifying potential molecules that could bind to residues 9–12 (PQLP) of  $\alpha$ 2-gliadin. The software used for this was Schrödinger 2015-4, employing the Maestro graphical interface. Databases of more than 5 million molecules were screened, including generally regarded as safe (GRAS) substances retrieved from the US Food and Drug Administration's (FDA) website. The screening of the GRAS database included 108 ligands, but after taking into account different tautomers, stereoisomers, and protonation states, the final database consisted of 1174 ligands. The free energy of binding was calculated for each ligand and the 17 substances with the lowest values were selected for evaluation using the transamidation assay.

#### 4.3.2. Transamidation assay

To solubilize gliadin (G3375; Sigma Aldrich, St. Louis, MO, USA), it was first manually ground, and then dissolved in ethanol (70%) and sonicated (3 × 5 min; Elmasonic S 15; Elma Schmidbauer GmbH, Singen, Germany) before coating on 96-well plates. The reason why whole gliadin was used instead of the smaller  $\alpha$ 2-gliadin (which was modeled previously) is that no satisfactory results could be obtained with the pure  $\alpha$ 2-gliadin, perhaps because it is too small to bind efficiently to the plate and also react with transglutaminase 2. The test molecules were then added and the rest of the transamidation assay was conducted in the same way as in Paper I. If the test molecules were to bind successfully to gliadin and block transglutaminase 2 transamidation, the signal detected would be weaker.

#### 4.3.3. Deamidation assay

A similar procedure for solubilizing gliadin and coating 96-well plates as used in the transamidation assay described above was used for the deamidation assay. For this assay, the only test molecule used was ascorbyl palmitate, by itself or together with zinc chloride ( $ZnCl_2$ ), since this was the only molecule that

showed positive results in the transamidation assay. Ascorbyl palmitate and  $\text{ZnCl}_2$  were allowed to react with the bound gliadin before adding transglutaminase 2. A mouse anti-deamidated gliadin antibody (ab36729; Abcam, Cambridge, UK) was used as a primary antibody to bind to deamidated gliadin, and a goat anti-mouse IgG antibody conjugated to horseradish peroxidase (A4416; Sigma Aldrich, St. Louis, MO, USA) was used as a secondary antibody for detection together with the Amplex ELISA development kit (Invitrogen, Paisley, UK). If ascorbyl palmitate were to bind successfully bind to gliadin and block transglutaminase 2 deamidation, the signal detected would be weaker.

#### 4.3.4. Cytotoxicity profile of ascorbyl palmitate in combination with gliadin

Based on positive results obtained in Paper II, in Paper III ascorbyl palmitate was further evaluated in Caco-2 cell cultures to assess its safety in combination with gliadin. The cells were grown at 37 °C in 5%  $\text{CO}_2$  with medium change every two to three days, and passaged at 80% confluence. Experiments were performed with cells at passage 34-49. Cells were plated in 12-well plates (200 000 cells/well) or 24-well plates (100 000 cells/well), and cultured for 14 days before the experiments. Gliadin, ascorbyl palmitate, and  $\text{ZnCl}_2$  were subjected to *in vitro* digestion as described in section 4.1 of this thesis, and then placed on the Caco-2 cell cultures for 3 hours.

Caco-2 is a human cell line originally obtained from a colon adenocarcinoma isolated by Fogh et al. in 1977 (132), and was provided by the American Type Culture Collection (HTB-37; ATCC, Manassas, VA, USA). Caco-2 cells originate from the colon, but if cultured to about five days post-confluence they spontaneously differentiate into cells that morphologically and functionally resemble mature duodenal enterocytes (133). Caco-2 cells are therefore extensively used as a model of the intestinal barrier for various application, such as intestinal permeability (134), effects of dietary compounds (135), cytokine responses from microorganisms (136, 137), cytotoxicity (138), and various celiac disease-related applications (78, 139, 140).

Four different aspects were examined to assess the safety of ascorbyl palmitate in combination with gliadin in Caco-2 cell cultures. First, the production of 12 inflammatory-related cytokines (IL1 $\alpha$ , IL1 $\beta$ , IL2, IL4, IL6, IL8, IL10, IL12, IL17A, IFN- $\gamma$ , TNF- $\alpha$  and GM-CSF) was measured using a commercial ELISA-based kit (MEH-004A; Qiagen N.V., Venlo, the Netherlands). Samples were incubated in sets in 96-well plates pre-coated with specific antibodies for each cytokine. A secondary biotinylated antibody, also specific to each cytokine, was added and finally Avidin-HRP, which binds to biotin, was used for detection. The most commonly measured cytokines when studying inflammatory responses in Caco-2 cell cultures are probably IL6 and IL8 (135, 137, 141-143). However, the 12 cytokines were selected to provide a broad assessment of possible inflammatory markers. Second, the level of cell survival was estimated through measuring the protein content with the bicinchoninic acid (BCA) protein kit. If any of the test samples were to induce cell death, less protein would be retrieved, as the culture wells were washed to remove cell debris before collecting the cell contents. Third, the cytotoxicity of the test samples was evaluated by measuring lactate dehydrogenase (Pierce 88954; Thermo Fisher Scientific, Rockford, IL, USA). Lactate dehydrogenase is a cytosolic enzyme and is thus released into the cell culture medium if the plasma membrane is damaged. The lactate dehydrogenase in the medium is used to catalyze the conversion of lactate to pyruvate by reducing  $\text{NAD}^+$  to NADH. The enzyme diaphorase subsequently uses the NADH to reduce a tetrazolium salt to formazan, which has an absorbance maximum at 490 nm. Caco-2 cells were plated on 96-well plates (12 000 cells/well) and cultured for 24 hours before adding the digested samples. After 3 hours, a portion of the medium was transferred to a new 96-well plate to start the reaction and measure the absorbance. Fourth, the integrity of the cell layers was assessed visually in six-well plates through a microscope (40x

magnification) after being subjected to digested samples for 3 and 24 hours. By combining these four methods, a robust evaluation of the cytotoxicity profile was made.

#### 4.4. Ancillary studies of human ileostomy contents

Gluten degradation *in vivo* has previously been studied in e.g., feces (144, 145) and in animal models (94), but not in human ileostomy samples. For studying human digestion up to the end of the ileum, human ileostomy samples are superior, since in feces the degradation is naturally strongly affected by the colonic microbiota and animal models may not fully represent human digestion. In this thesis, gluten digestion was investigated in human ileostomy samples collected and described previously (146, 147). The aim of the original studies was to investigate the effect on digestibility of primarily starch, dietary fiber, phytate, and the absorption of nutrients after intake of a high-fiber cereal product eaten raw or in extruded form. The ileostomy contents have since been stored freeze-dried at -20 °C. The cereal product studied also contained 10% gluten, which makes these ileostomy samples suitable for studying how gluten is digested in the stomach and the small intestine in humans *in vivo*.

##### 4.4.1. Original study design

Over two four-day periods, individuals with well-functioning ileostomies (positioned at the end of the ileum) were given a constant low-fiber diet supplemented with either 54 g/day of a bran-gluten-starch mixture or the corresponding extruded product. The mixture consisted of 60% starch, 30% bran, and 10% gluten, and was ingested half with lunch and half with dinner. The ileostomy bags were changed at regular intervals (every 2 hours) and immediately frozen. The contents were freeze-dried, homogenized, and pooled for each study day.

##### 4.4.2. Protein, $\alpha$ -gliadin, and microstructure of ileostomy samples

The freeze-dried samples were re-suspended in tissue lysis buffer, centrifuged to remove particulates, and the supernatant was used for further analysis. The total protein content was determined using the BCA protein kit and the results were used for loading equal amounts of protein for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) used for determination of protein size distribution and in a Western blot methodology. The primary antibody used binds to a 16 amino acid long segment of the  $\alpha$ -gliadin peptide, which is part of  $\alpha$ -gliadin (ab36729; Abcam, Cambridge, UK). Bright field microscopy was performed by Dr Karin Autio, formerly at VTT Technical Research Centre of Finland Ltd, to visualize the microstructure of the digested samples.

## 5. RESULTS AND DISCUSSION

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### 5.1. Lactic acid fermentation of wheat flour

Fermentation with *Lactobacillus plantarum* increased the levels of  $\alpha$ 2-gliadin in the fermented wheat flours, detected by a specific antibody, by  $22 \pm 18 \%$  ( $p=0.047$ ) compared with the unfermented control, suggesting that more substrate was accessible for transglutaminase 2 after the fermentation. However, this was not the outcome indicated by the transamidation assays, which showed a decrease in transamidation of gluten by *L. plantarum* ( $27 \pm 18 \%$ ,  $p<0.001$ ). Thus, *L. plantarum* seems to be able to degrade some parts of gluten, but not  $\alpha$ 2-gliadin. The decrease of 27% compared with the control is not very impressive, however, especially considering that the fermentation had taken place in a fairly dilute solution of wheat flour for almost 24 hours. The increased accessibility of  $\alpha$ 2-gliadin might increase the immune response in celiacs. In contrast, in the sourdough breads the recognized  $\alpha$ 2-gliadin level, fermented by *L. plantarum*, was decreased by  $19 \pm 15 \%$  ( $p=0.042$ ). The fermentation of the wheat flour was more extensive than that of the wheat flour in the sourdough bread (100% of the flour fermented 21 hours in a dilute slurry, compared with 24 hours as a sourdough and 20% added to a bread dough fermented for 4 additional hours). Based on the results from the fermented wheat flour, it was expected that  $\alpha$ 2-gliadin levels would lie between that increase and the control. However, these results suggest that a more extensive fermentation with *L. plantarum* produces more accessible  $\alpha$ 2-gliadin peptides.

Fermentation of wheat flours with *Lactobacillus pentosus*, on the other hand, decreased  $\alpha$ 2-gliadin by  $27 \pm 21 \%$ , ( $p=0.027$ ), but there was no significant change at all for the transamidation ( $+2 \pm 25 \%$ ,  $p=0.564$ ). This indicates that, even though the total binding of transglutaminase 2 to gluten was unaffected, this species has some capacity for degrading  $\alpha$ 2-gliadin. However, as with the gluten-degrading capacity of *L. plantarum*, the decrease was quite small and far from enough to yield a safe food product for celiacs. *Lactobacillus pentosus* did not differ from the control in accessibility of  $\alpha$ 2-gliadin in the sourdough breads. *Lactobacillus brevis* showed no significant differences compared with the control for either the fermented wheat flour slurry or for the sourdough breads. The results from all experiments are compiled in Figure 4.

A combination of *L. plantarum* and *L. pentosus* could perhaps reduce both the amount of accessible  $\alpha$ 2-gliadin and the total transglutaminase 2 interaction with gluten. This combination has not been tested, but with these species it is unlikely that even a combination would be able to degrade all immunogenic peptides fully under similar conditions. As mentioned previously, these species were not chosen in this thesis for their ability to degrade gluten. In other studies where this has been done, it has been shown that it is possible to degrade gluten, but that this requires long fermentation times and also addition of proteases (11, 148). Moreover, the results obtained here show that a general lactic acid fermentation that is not carefully tailored to degrade gluten is unlikely to be sufficiently beneficial for celiacs to be used as therapy. Instead of being helpful, partial gluten degradation may actually create more available immunogenic peptides and shorter peptides that can enter the lumen more easily.

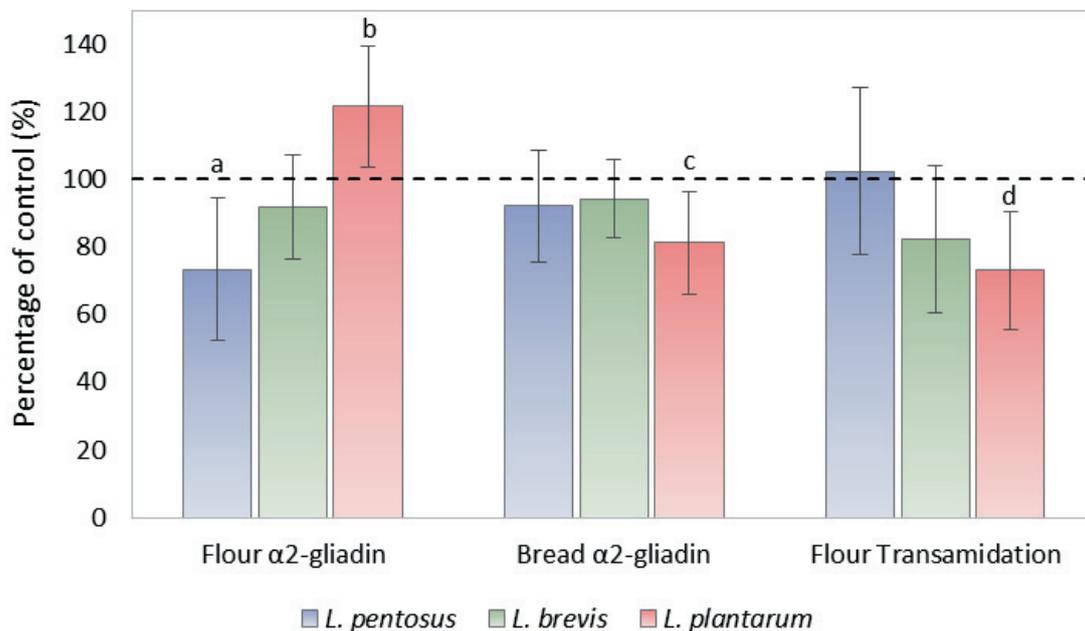


Figure 4. Combined results for available  $\alpha$ 2-gliadin and transamidation, using either fermented wheat flour or sourdough bread with different *Lactobacillus* species. All values are related to their respective control and presented as percentage of control. The lower case letters indicate significant *p* values (significance set at  $p < 0.05$ ). a:  $p = 0.027$ , b:  $p = 0.047$ , c:  $p = 0.042$ , d:  $p < 0.001$ .

## 5.2. Preventing transglutaminase 2-gliadin interactions through binding molecules

### 5.2.1. Computational modeling and screening of docking molecules for $\alpha$ 2-gliadin

A homology model of  $\alpha$ 2-gliadin was created based on the five templates obtained from the RSBC Protein Data Bank (Figure 5). This model was used for screening a large set of molecules, including 108 GRAS molecules (in total 1174 entries). Of these, the five best ligands were chosen based on their binding energies, but also on their acceptable daily intake levels and sensory properties. Another 12 molecules that had been chosen prior to the simulation were also included (see Table 2).

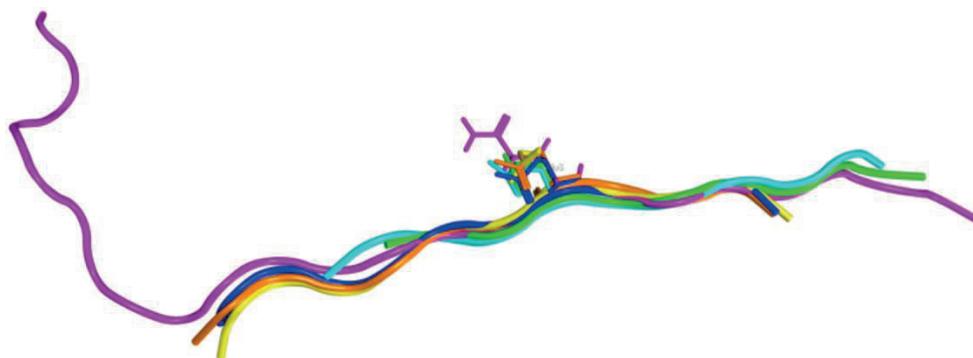


Figure 5. The final homology model of  $\alpha$ 2-gliadin created (magenta), superposed with the five templates.

Two molecules ranked higher than the chosen molecules with regard to binding energy, but were excluded due to the very low solubility of magnesium stearate and due to polyethylene glycol monostearate being a common ingredient in e.g., soaps and shampoos, which could lower consumer acceptance. Additionally, magnesium stearate interacts with the QPFPQPQLP sequence, which is only present once in  $\alpha$ 2-gliadin, and thus would not cover all three transglutaminase 2 binding motifs present in  $\alpha$ 2-gliadin. Ascorbyl palmitate, on the other hand, which ranked highest based on binding energy among the chosen molecules, interacts with the QLPYPQ sequence and could therefore, at least in theory, cover every transglutaminase 2 binding motif on  $\alpha$ 2-gliadin.

*Table 2. Simulated binding energies and in vitro transamidation results for selected ligands screened against the motif (P)QLP in the homology model of  $\alpha$ 2-gliadin. The ranking is based on binding energy. Transamidation is presented as a percentage of control. In the transamidation screening, the compounds were used at 15  $\mu$ M.*

Rank	Compound	Binding energy (MMGBSA dG Bind)	Transamidation screening (% of control)
1	Ascorbyl palmitate	-52.790	30
2	Taurocholic acid	-51.688	102
3	Ergocalciferol	-48.361	93
4	Dextran	-41.784	96
5	Sodium stearate	-37.831	94
6	Glyceryl diacetate	-27.114	103
7	Carnitine (L)	-25.935	106
8	Glutathione (red)	-24.207	94
9	Adipic acid	-20.950	106
10	Carnosine (L)	-20.152	106
11	Glyceryl triacetate	-18.783	106
12	Lysine (L)	-17.967	108
13	Cysteine (L)	-13.738	100
14	Lactate	-11.578	98
15	Taurine	-11.564	98
16	Succinic acid	-10.330	111
17	Tartaric acid	-9.210	96

### 5.2.2. Ascorbyl palmitate decreases trans- and deamidation of gliadin

As previously described, whole gliadin was used in the assays, due to difficulties in obtaining reliable results using  $\alpha$ 2-gliadin. Apart from the three binding motifs contained in the  $\alpha$ 2 part, whole gliadin contains six additional transglutaminase 2 binding motifs. The ability of test molecules to bind to these other motifs was not simulated.  $\alpha$ 2-gliadin is considered to be the most immunogenic peptide in celiac disease, both because it contains three sites for transglutaminase 2 and because this peptide is highly resistant to gastrointestinal degradation.

The 17 chosen molecules were first tested in a single concentration (15  $\mu$ M) as a second screening step using the transamidation assay (see Table 2). Using this concentration, ascorbyl palmitate was the only molecule that could lower the level of transamidation compared with the control to a major extent (70  $\pm$  7 %,  $p < 0.05$ ). Ascorbyl palmitate (AP) was further investigated in dose-response experiments in

concentrations ranging from 5-60  $\mu\text{M}$ , both on its own and together with zinc chloride (Figure 6). With 30  $\mu\text{M}$ , the decrease reached a plateau ( $80 \pm 3\%$ ,  $p < 0.05$ ) and the next concentration at 60  $\mu\text{M}$  was not significantly different ( $82 \pm 2\%$ ,  $p = 0.061$ ) compared with 30  $\mu\text{M}$ . With the addition of small amounts of  $\text{ZnCl}_2$  (up to 50  $\mu\text{M}$ ), the decrease was minimized even further ( $93 \pm 2\%$  using 60  $\mu\text{M}$  AP and 50  $\mu\text{M}$   $\text{ZnCl}_2$ ), reaching lower levels compared with using  $\text{ZnCl}_2$  alone ( $63 \pm 6\%$ ). With the addition of higher amounts of  $\text{ZnCl}_2$  (up to 1000  $\mu\text{M}$ ), the level of transamidation was close to zero, but with the highest concentration of  $\text{ZnCl}_2$  the contribution of ascorbyl palmitate was minor. Ascorbyl palmitate and  $\text{ZnCl}_2$  work synergistically at lower levels of  $\text{ZnCl}_2$  but at higher concentrations of  $\text{ZnCl}_2$  its transglutaminase 2 inhibiting effect takes over.

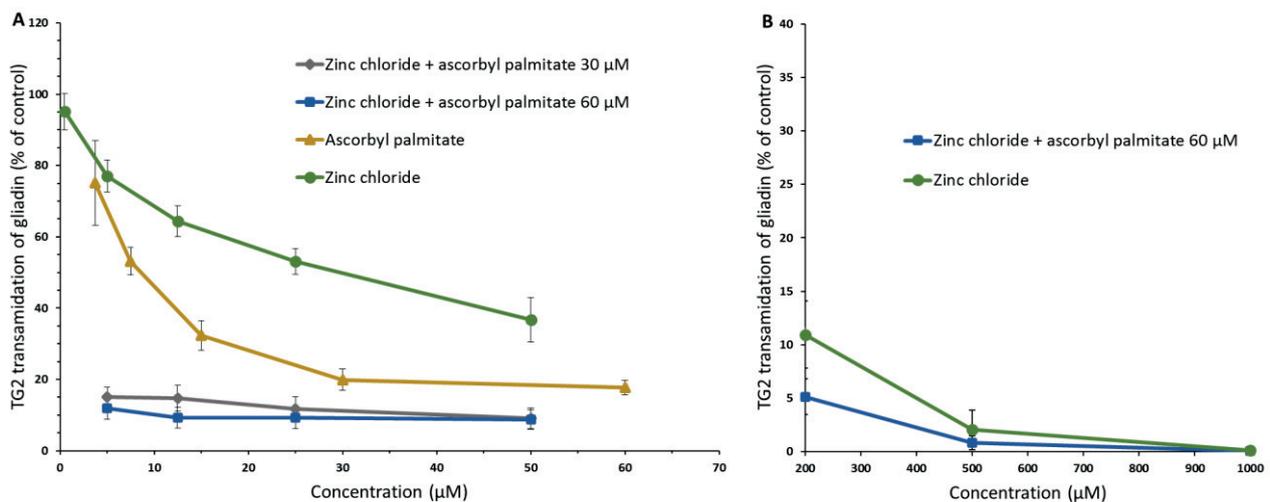


Figure 6. Transglutaminase 2 (TG2)-mediated transamidation of gliadin in dose response experiments using ascorbyl palmitate and zinc chloride. (A) Dose response of ascorbyl palmitate and zinc chloride used singly, and dose response of zinc chloride in combination with ascorbyl palmitate at 30 and 60  $\mu\text{M}$ . (B) Zinc chloride (200-1000  $\mu\text{M}$ ) used singly and in combination with ascorbyl palmitate at 60  $\mu\text{M}$ . With 500  $\mu\text{M}$  zinc chloride and 60  $\mu\text{M}$  ascorbyl palmitate, TG2 transamidation was completely inhibited. Values presented are mean  $\pm$  SD ( $n=3$ ).

Ascorbyl palmitate was also examined in the ELISA-based deamidation assay. Plate-bound gliadin was subjected to transglutaminase 2 without addition of any primary amine, with or without addition of ascorbyl palmitate and  $\text{ZnCl}_2$ . The mouse anti-deamidated gliadin antibody was used to bind deamidated gliadin and goat anti-mouse IgG antibody conjugated to horseradish peroxidase was used for detection. Transglutaminase 2-treated gliadin had significantly higher deamidation compared with gliadin in combination with ascorbyl palmitate. Furthermore, no significant difference could be seen for gliadin in combination with ascorbyl palmitate treated with transglutaminase 2 compared with native gliadin (results not shown). One important point to note here is that the concentrations used are difficult to translate outside these experiments. They depend on e.g., the volume used, incubation times, the amount of gliadin bound to the plate, and the amount of transglutaminase 2 used. The amount of gliadin added is known, but not how much has actually bound to the plate and how much has washed away.

### 5.2.3. Ascorbyl palmitate and zinc as food additives

Ascorbyl palmitate is used as a food additive (E 304i) mainly in lipid-rich foods. It is permitted for use *quantum satis*, which means that there is no definitive restriction on amount, but no more than is needed should be added. In the USA, ascorbyl palmitate is labeled as a GRAS substance and approved by the Food and Drug Administration (US FDA) as a food additive. The primary function of ascorbyl palmitate as a food

additive is as an antioxidant, in the same manner as ascorbic acid (vitamin C), but with a higher solubility in lipids. It is commercially produced by esterification of ascorbic acid with palmitic acid in concentrated sulfuric acid (149, 150). Ascorbyl palmitate can also be produced by enzymatic synthesis using a lipase (151, 152). In 1973, The Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) has evaluated ascorbyl palmitate and has set an acceptable daily intake of 1.25 mg/kg body weight (bw)/day (153). This intake is based on a study in rats using up to the equivalent of 125 mg/kg bw/day of ascorbyl palmitate, without observing any adverse effects. A safety factor of 100 has been set, leading to the acceptable daily intake of 1.25 mg/kg bw/day (153). In 2015, the European Food Standards Agency (EFSA) evaluated ascorbyl palmitate as a food additive and found no safety concerns with its use, with an estimated mean intake of ascorbyl palmitate (in Europe) of 0.2-3.2 mg/kg bw/day, or 0.4-10.8 mg/kg bw/day for the highest percentile of the population (149). As raw materials, ascorbyl esters are sensitive to oxygen and light, and should preferably be stored in dark, sealed containers. It is assumed that ascorbyl palmitate is pre-systemically hydrolyzed into ascorbic acid and palmitic acid during digestion (149). However, the digestive stability of ascorbyl palmitate in combination with gliadin has not been investigated, but could be assumed to be at the same level or higher than for ascorbyl palmitate on its own. Ascorbyl palmitate can be used as a dough conditioner and anti-staling agent in bread instead of the more thermolabile ascorbic acid (154). In bread containing ascorbyl palmitate, roughly 80% can be recovered after baking, showing that ascorbyl palmitate is resistant to the high temperatures of baking (154, 155). Ascorbyl palmitate is also a strong promoter of iron absorption (155). Thus, ascorbyl palmitate has several beneficial properties and has already been shown to be useful in bread making.

The amount of  $Zn^{2+}$  needed *in vivo* to obtain the same result is dependent on the amount of intestinal transglutaminase 2, which is likely to be less than the concentration used in these experiments, as well as the intestinal concentration of  $Ca^{2+}$ . The amount of  $Zn^{2+}$  needed cannot be determined in this type of *in vitro* experiment, but must be further investigated *in vivo*. The dietary requirement for zinc is closely related to body weight, but also to the intake of phytate, which has an inhibitory effect on zinc absorption (156). Depending on these two factors, EFSA recommends for adults a dietary intake of zinc of roughly 6-16 mg/day (156). The Swedish National Food Agency (Livsmedelsverket) and the Nordic Nutrition Recommendations (2012) have set the recommended daily intake of zinc to 7 mg for women and 9 mg for men. Chronic excess intake of zinc can lead to a decreased copper uptake, which may lead to severe neurological diseases (156). The Nordic Nutrition Recommendations (2012) and EFSA have both set an estimated upper intake level of 25 mg, which taken daily would be unlikely to pose a risk of adverse health effects in a normal healthy person. The EFSA also reports a "No Observed Adverse Effect Level" of 50 mg/day, as this level does not seem to affect copper status. Zinc acetate (E 650) is allowed in chewing gum to a maximum of 1000 mg/kg. Zinc acetate and several other zinc salts, including zinc chloride and zinc gluconate, are permitted in dietary supplements. The typical dose in supplements is 7-15 mg/day, but there are supplements available with a concentration of up to 50 mg/day. Zinc is a very effective inhibitor of transglutaminase 2 and, considering also its anti-inflammatory properties and ability to improve regeneration of damaged gut epithelium, it might be beneficial to add zinc to gluten-containing products to prevent initiation of celiac disease (76, 108, 112).

#### 5.2.4. Cytotoxicity profile of ascorbyl palmitate in cell cultures

The cytotoxicity profile of ascorbyl palmitate in combination with gliadin was assessed in Caco-2 cell cultures by examining four different aspects, three of which focus on cell death (cytotoxicity, cell survival,

and cell layer integrity) and one on cellular inflammatory markers (cytokines). None of the aspects evaluated showed any negative effects of ascorbyl palmitate, when used either alone or in combination with gliadin or ZnCl<sub>2</sub> (5-10 µM; results not shown).

Cytotoxicity (Figure 7) and cell survival (Figure 8), estimated by measuring total protein content, were both evaluated after cell exposure to gliadin (0.25 µg/mL) and concentrations of ascorbyl palmitate ranging from 0-10 µg/mL. However, for cytotoxicity the second highest concentration of ascorbyl palmitate (7.5 µg/mL) was significantly higher than gliadin alone (6.3% compared with 1.6%, p=0.034). Cell layer integrity was not visually affected after either 3 or 24 hours of incubation with gliadin and the highest concentration of ascorbyl palmitate (10 µg/mL) (results not shown).

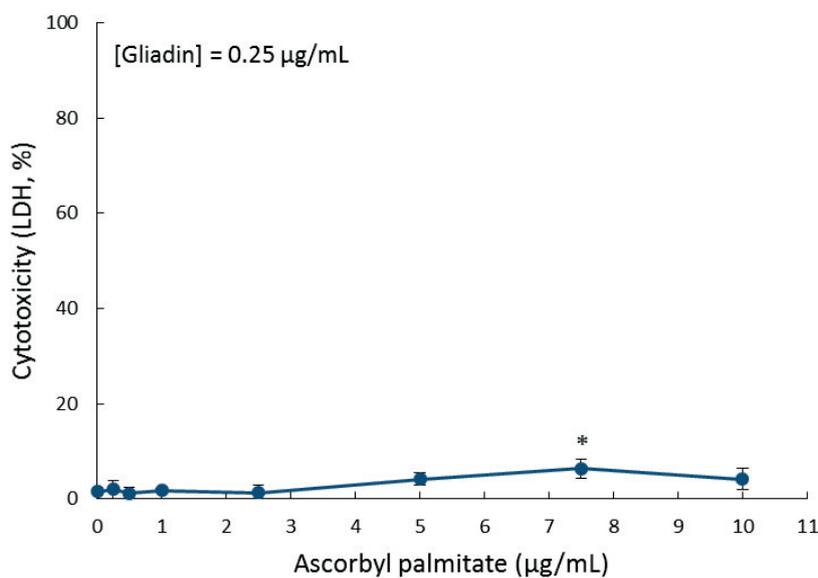


Figure 7. Cytotoxicity evaluation in Caco-2 cells after exposure to digests of gliadin (0.25 µg/mL) and ascorbyl palmitate (0-10 µg/mL). Values presented are mean ± SD (n=3). \*4.7% points, p=0.034.

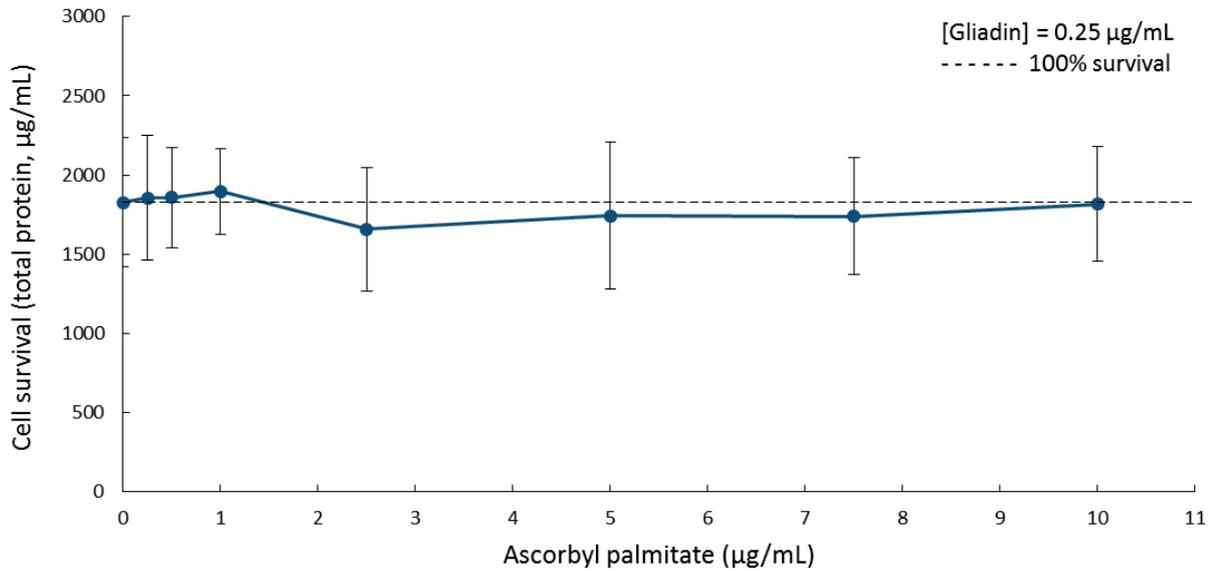


Figure 8. Cell survival of Caco-2 cells exposed to digests of gliadin (0.25 µg/mL) and ascorbyl palmitate (0-10 µg/mL). Cell survival estimated based on the amount of protein compared with a control (100%). Values presented are mean ± SD (n=6).

Production of inflammatory-related cytokines was assessed in both lysed cells and when released into the culture medium after exposure to gliadin (0.25 µg/mL) and the highest concentration of ascorbyl palmitate (10 µg/mL) (Figure 9). Altogether, the results indicate that the combination of ascorbyl palmitate and gliadin is well tolerated in Caco-2 cell cultures and that ascorbyl palmitate should be further investigated as a potential food additive for making gluten-containing foods safe for celiacs.

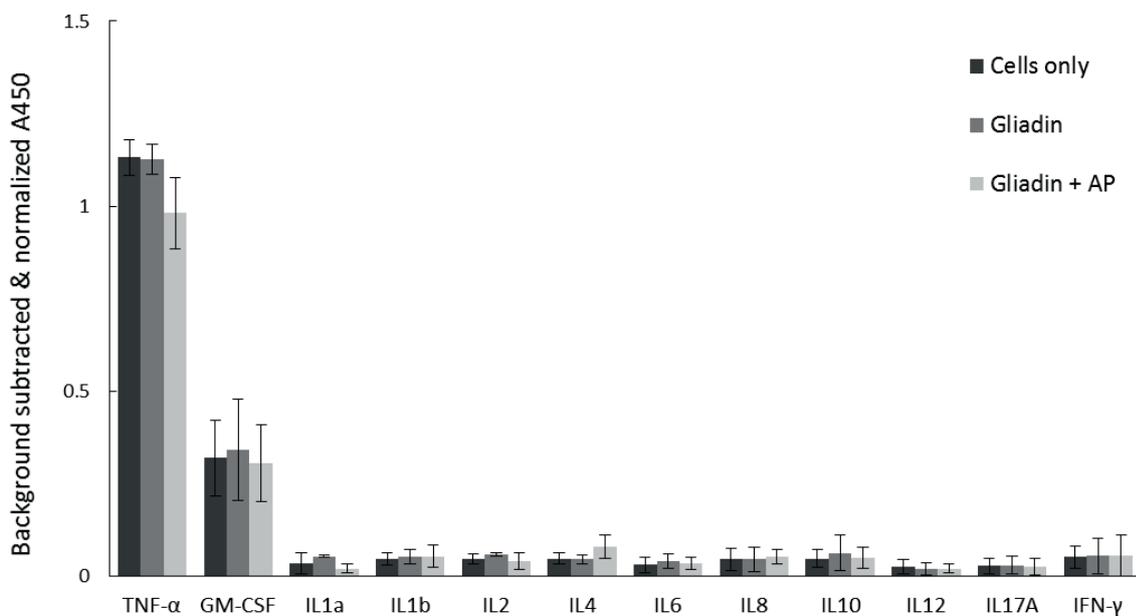


Figure 9. Cytokines in Caco-2 cell lysates after exposure to digests of gliadin (0.25 µg/mL), gliadin (0.25 µg/mL) and ascorbyl palmitate (10 µg/mL), compared with untreated cells. Values presented are mean ± SD (n=3).

Due to the poor solubility of gliadin in water, combined with the relatively mild *in vitro* digestion used here, it was difficult to obtain homogeneous samples when using higher concentrations of gliadin. Here, the gliadin concentration was set to 0.25 µg/mL, which can be compared with commonly used concentrations of between 0.5-1 mg/mL in other studies (50, 51, 157, 158). In those studies, and others, the digestion protocols are typically much harsher, with longer incubation times using both pepsin and trypsin, simulating the whole small intestine, and also including 10-30 min heating at 90-100 °C to inactivate the trypsin (50, 51, 157, 158). As mentioned in section 4.1, the reason for the shorter digestion used throughout this thesis is that gluten peptides may cause an immune response as soon as they enter the small intestine. Moreover, even though inactivation of trypsin by heating is commonly used, it is not especially physiologically correct and is likely to affect the samples in other ways than just inactivating trypsin.

### 5.3. Ancillary studies of human ileostomy contents

Based on these preliminary results, the protein content was 30-50% higher in the ileostomy contents derived after intake of the extruded product, suggesting that the extrusion process used here leads to decreased protein digestibility. The decreased digestibility was further supported by the bright field microscopy work (Figure 10), which indicated that the extrusion process preserves the microstructure, making it more resistant to degradation. The aleurone layer of the bran was highly conserved in the extruded product after digestion, compared with the almost complete degradation seen in the raw product. Decreased protein digestibility has also previously been demonstrated after thermal treatment (159-161), but also the reversed have been shown for protein digestibility after extrusion (162, 163). However, the size distribution of the proteins was similar between the raw and extruded product after digestion (Figure 11). If the extrusion process had decreased protein digestibility, a larger proportion of proteins with higher molecular weight, and less with lower molecular weight, could be expected compared with the raw product.

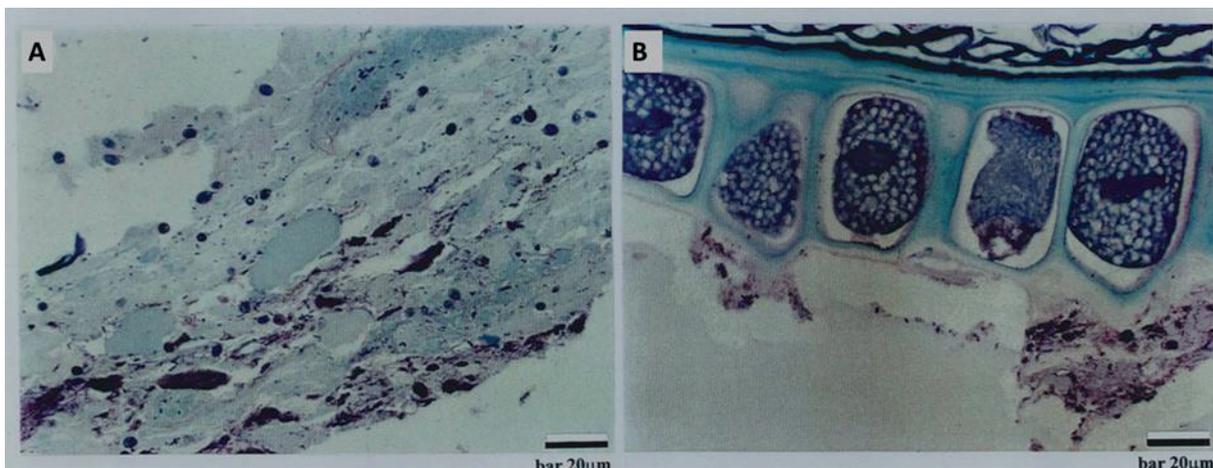


Figure 10. Bright field microscopy of embedded section of ileostomy contents: (A) After intake of raw product, where yeast cells appear as blue deposits, and (B) after intake of extruded product, where an intact aleurone layer can be seen. Photos taken by Dr Karin Autio, VTT, Finland.

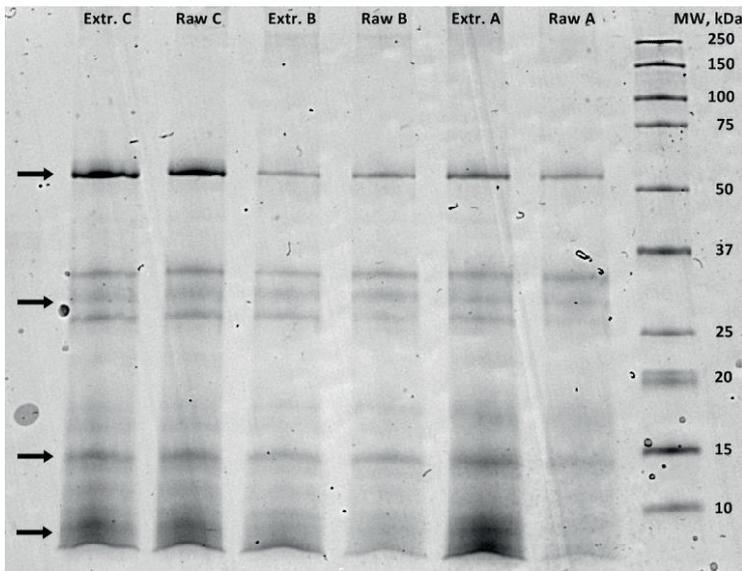


Figure 11. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein-normalized, in vivo digested, raw or extruded product, in three ileostomy subjects (A, B, and C). A 40  $\mu$ g portion of total protein was loaded in each lane. Coomassie blue was used for the staining.

Intact  $\alpha$ -gliadin was detected in samples obtained after intake of the raw and the extruded product in both ileostomy subjects investigated (Figure 12). The strong bands in the center probably correspond to intact  $\alpha$ -gliadin and the fainter, extended bands seen underneath the intact  $\alpha$ -gliadin probably correspond to partially degraded  $\alpha$ -gliadin that still contains the amino acid sequence specific for the antibody used. The amount of  $\alpha$ -gliadin is substantially lower in the extruded product. However, the same amount of total protein was loaded for each sample, and the extruded product contained 40-50% more protein for these samples. Thus, the extrusion might have increased digestion of  $\alpha$ -gliadin, but there is also the possibility that proteins other than  $\alpha$ -gliadin are digested to a lower extent due to the extrusion process leading to the lower proportion of  $\alpha$ -gliadin seen in Figure 12.

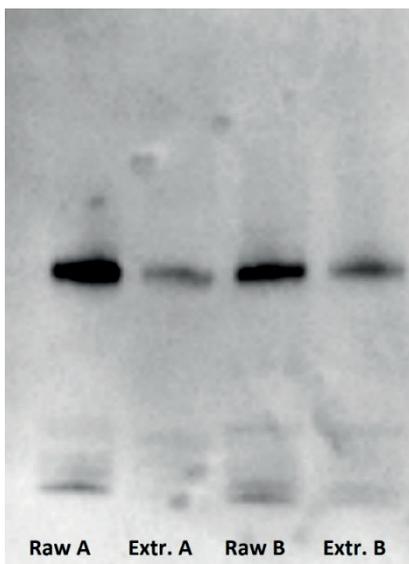


Figure 12. Western blot of protein-normalized, in vivo digested, raw or extruded product, in two ileostomy subjects (A and B). A 40  $\mu$ g portion of total protein was loaded in each lane and  $\alpha$ -gliadin peptides were detected.

## 6. CONCLUSIONS

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This thesis investigated several strategies for preventing the interaction between transglutaminase 2 and gluten in the human intestine. One of the strategies, which involved blocking the binding motifs for transglutaminase 2 on gluten peptides, produced promising results. Based on the results of all four studies, the following conclusions can be drawn:

- Standard lactic acid fermentation used in commercial preparation of sourdough bread is not likely to be safe for celiacs. Fermentation can be used successfully to degrade gluten, but that requires long and/or complex fermentations and possibly addition of proteases. Furthermore, complete degradation of gluten will automatically reduce the quality, as the beneficial properties of gluten are lost.
- Ascorbyl palmitate interacts strongly with transglutaminase 2 binding motifs on  $\alpha$ 2-gliadin *in silico*. Moreover, it is able to significantly decrease the transglutaminase 2 interaction to gliadin *in vitro*. When acting in synergy with zinc chloride, ascorbyl palmitate can completely prevent interaction between transglutaminase 2 and gliadin.
- Ascorbyl palmitate, by itself or in combination with gliadin or zinc chloride, evokes no negative effects on the cytotoxicity profile in human intestinal Caco-2 cell cultures.
- Ascorbyl palmitate, in combination with zinc chloride, shows promise as an additive to flour products for creating celiac-safe foods.
- A larger proportion of intact  $\alpha$ -gliadin compared with  $\alpha$ 2-gliadin-containing peptides can be detected after human *in vivo* digestion, suggesting that whole  $\alpha$ -gliadin is resistant to gastrointestinal digestion. Preliminary results indicate that the extrusion process used here decrease the protein digestibility of a bran-gluten-starch product, as well as maintaining the microstructure to a higher extent after human *in vivo* digestion.

## 7. FUTURE PERSPECTIVES

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- Ascorbyl palmitate has so far only been tested with isolates of gliadin. It is necessary also to perform experiments with real flour, in a product such as a bread, to establish that the preventive effect of ascorbyl palmitate on transglutaminase 2 interaction to gliadin is maintained.
- A celiac disease-related immune response can potentially occur early in the small intestine, and therefore the *in vitro* digestions used in this thesis ended at the start of the duodenum. However, it is also necessary to evaluate the stability of the combination of ascorbyl palmitate and gliadin to further degradation, since an immune response might be triggered at a later stage, during gastrointestinal digestion.
- T-cells or intestinal biopsies from individuals with celiac disease could be used to test products treated with ascorbyl palmitate, and ascorbyl palmitate together with zinc, to assess the effectiveness in preventing celiac disease-specific reactions.
- Ascorbyl palmitate and zinc could be further investigated in a gluten-containing product in animal models and clinical trials with individuals with celiac disease, to evaluate the usefulness *in vivo*.

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