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Digestion of gluten in wheat sourdough compared to wheat flour, in normal persons and celiac patients.

Fordøyelse av gluten i hvetesurdeig sammenlignet med hvetemel, i normale personer og cøliaki pasienter.

Trine Aarnes Tessem

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Abstract

The only treatment of celiac disease today is a strict life-long gluten-free diet. Gluten is the plays a unique role in the wheat's baking quality. Making bread without gluten is difficult and expensive. In recent years sourdough has been tried out, hoping to develop a good quality bred tolerated by celiac patients. The aim of this study was to detect differences in the protein profile and the content immune reactive amino acid sequences (mg/kg) of the gluten in wheat flour and four wheat sourdoughs (with different starter cultures) before and after *in vitro* digestion, using human gastrointestinal enzymes from normal persons and from two individual celiac patients. The protein profiles were analyzed with SDS-PAGE, and ELISA analyzed the content immune reactive amino acid sequences (mg/kg) in gluten proteins and peptides.

The protein profile of the wheat sourdoughs showed that one to two protein bands might be missing, compared to the wheat flour. But, the content mg/kg of immune reactive gluten sequences was the same for the wheat flour and wheat sourdoughs, 597,7mg/kg. The Norwegian food authority (Mattilsynet) have set the content of gluten proteins to <100mg/kg and <20mg/kg to label a product *low in gluten* or *gluten-free* respectively. With the content detected with ELISA in this study, the wheat sourdoughs cannot be labelled *low in gluten* or *gluten-free.*

The digestion of wheat flour and the wheat sourdoughs with gastric juice from normal persons was done at pH2 and pH4. The protein profiles showed that all proteins were digested into smaller peptides Mw <14,4kDa. The wheat flour digested at pH2 had a content of 87 mg/kg immune reactive gluten sequences, compared to 184mg/kg after digested at pH4. The wheat sourdoughs digested with gastric juice at pH2 had content between 13-37mg/kg, compared to the content between 46-89mg/kg after digested at pH 4. After digested with duodenal juice at pH7 all the samples had content <14mg/kg.

Wheat flour and wheat sourdough 3 were digested with gastrointestinal enzymes from celiac patients. The digestion with the gastric juice from the one celiac patient was done at pH7,4 (normal pH for the celiac patient) and pH2. The protein profile of the wheat flour digested with gastric juice pH7,4 from the celiac patient showed that all protein Mw >14,4kDa

remained undigested, while the protein profile of digested wheat sourdough 3 showed that most of the proteins Mw >14,4kDa remained undigested. The wheat flour digested with pH 7,4 had content of 505mg/kg immune reactive gluten sequences, and a content of 511mg/kg after digested at pH2. The wheat sourdough 3 digested at pH7,4 had a content of 396mg/kg, and a content of 379mg/kg after digested at pH2. After the duodenal digestion all samples had content <20mg/kg.

The protein profiles and the ELISA results in this study showed that the gastric juice from normal persons digested a higher content of immune reactive gluten sequences, compared to the celiac patient. They also showed that after digested with gastric juice from normal persons and the one celiac patient, the content of immune reactive gluten sequences was lower in all the wheat sourdough samples, compared to the wheat flour samples. This showed that the immune reactive gluten sequences in the wheat sourdoughs were easier to digest. After further digested with duodenal juice the content had decreased, but in almost all samples a small amount immune reactive gluten sequences were still detected, which indicated that the gastrointestinal enzymes wasn't able to digest the gluten proteins and peptides completely.

Sammendrag

Den eneste behandling av cøliaki i dag er en streng livslang glutenfri diett. Gluten spiller en unik rolle i bakekvaliteten til hvete. Å lage brød uten gluten er vanskelig og kostbart. I de senere årene har surdeig blitt prøvd ut i håp om å utvikle et brød med god kvalitet som tolereres av cøliakipasienter. Målet med denne studien var å detektere forskjeller i proteinprofilen og innhold av immun reaktiv aminosyresekvenser i gluten protein og peptider (mg/kg) i hvetemel og fire hvetesurdeiger (med forskjellige startkulturer), før og etter *in vitro* fordøyelse med humane gastrointestinale enzymer fra normale personer og to individuelle cøliakipasienter. Proteinprofilene ble analysert med SDS –PAGE, og ELISA analyserte innhold immunreaktive aminosyresekvenser i glutenproteiner og peptider (mg/kg) .

Proteinprofilen til hvetesurdeigene viste at ett til to proteinbånd kan hende manglet, sammenlignet med hvetemel. Men, innholdet mg/kg av immunoreaktive glutensekvenser var den samme for hvetemel og hvetesurdeigene, 597,7 mg/kg. Det norske Mattilsynet har satt <100mg/kg and <20mg/kg som grenseverdier for å kunne merke et produkt som følgende, *lavt innhold av gluten* eller *glutenfritt*. Mengdeverdiene målt med ELISA i denne studien tilsier at hvetesurdeigene ikke kan bli merket med *lavt innhold av gluten* eller *glutenfritt*.

Fordøyelsen av hvetemel og hvetesurdeigene med magesaft fra normale personer ble gjort ved pH2 og pH4. Protein profilene viste at alle proteiner ble fordøyd til mindre peptider Mw <14,4 kDa. Hvetemelet fordøyd ved pH2 hadde et innhold på 87mg/kg immunoreaktive gluten sekvenser, sammenlignet med 184mg/kg etter fordøyd på pH4 . Hvetesurdeigene fordøyd med magesyre ved pH 2 hadde et innhold mellom 13-37mg/kg, sammenlignet med innholdet mellom 46-89mg/kg etter fordøyd ved pH 4. Etter fordøyelse med duodenal juice ved pH7, hadde alle prøvene et innhold < 14mg/kg .

Hvetemel og hvetesurdeig 3 ble fordøyd med gastrointestinale enzymer fra cøliakipasienter. Fordøyelsen med magesaften fra en cøliaki pasient ble utført ved pH7,4 (normal pH for cøliaki pasienten) og pH2. Proteinet profilen til hvetemelet fordøyd med magesaft pH7,4 fra cøliaki pasient viste at alle proteiner Mw >14,4 kDa forble ufordøyd, mens proteinprofilen til hvetesurdeig 3 fordøyd med magesaft med pH 7,4 viste at mesteparten av proteinene Mw > 14,4 kDa forble ufordøyd. Hvetemel prøven etter fordøyelse ved pH 7,4 innholdet av 505mg/kg immunoreaktive glutensekvenser, og 511mg/kg fordøyelse ved PH2. Hvetesurdeig

3 fordøyd ved pH7,4 hadde et innhold på 396mg/kg, og et innhold på 379mg/kg etter fordøyelse ved pH2 . Etter duodenal fordøyelse med duodenalsaft fra en cøliaki pasient inneholdt alle prøvene < 20mg/kg .

Protein profilene og ELISA resultatene i denne studien viste at magesaften fra normale personer fordøyde større mengde mg/kg immunoreaktive gluten sekvenser enn cøliaki pasienten. De viste og så at etter fordøyelse med magesaft fra normale personer og den ene cøliaki pasienten, var innholdet mg/kg av immun reaktive gluten sekvenser lavere i alle hvete surdeig prøvene, sammenlignet med hvetemel prøvene. Dette viste at immunoreaktive glutensekvenser i hvetesurdeigene var lettere å fordøye i magesaften. Etter ytterligere fordøyelse med duodenalsaft var innholdet redusert, men i nesten alle prøvene ble det fortsatt detektert en liten mengde immunoreaktive gluten, noe som indikerte at de gastrointestinale enzymer var ikke i stand til å fordøye glutenproteinene og peptidene fullstendig.

Table of contents

ACKNOWLEDGMENTS ..... **I**

ABSTRACT ..... **II**

SAMMENDRAG ..... **IV**

1 INTRODUCTION **1**

1.1 WHEAT 1

1.1.1 Gluten *2*

1.1.2 Gluten proteins function in bread *3*

1.1.3 Proteins related to wheat gluten *4*

1.2 CELIAC DISEASE 5

1.3 THE HUMAN DIGESTIVE SYSTEM AND ITS ENZYMES 7

1.3.1 Mouth, pharynx and esophagus *8*

1.3.2 The ventricle *8*

1.3.3 Small intestine *9*

1.3.4 The large intestine *11*

1.3.5 Immune reactive gluten peptides *11*

1.3.6 Digestion of gluten *12*

1.4 SOURDOUGHS 13

1.5 TREATMENT OF CELIAC DISEASE 14

1.6 AIM OF STUDY 15

2 MATERIALS AND METHODS **16**

2.1 MATERIALS 16

2.1.1 Wheat flour *16*

2.1.2 Wheat sourdoughs *16*

2.1.3 Human Gastric and duodenal juice *16*

2.2 GLUTEN EXTRACTION PROCEDURES 16

2.2.1 Ethanol (60%) extraction method *17*

2.2.2 UPEX extraction method *17*

2.3 MEASURING PROTEIN CONTENT 17

2.3.1 Kjeldahl method *17*

2.4 PH IN THE HUMAN GASTRIC JUICES AND THE HUMAN DUODENAL JUICES. 18

2.5 PROTEOLYTIC ACTIVITIES OF GASTRIC AND DUODENAL ENZYMES 19

2.6 *IN VITRO* DIGESTION OF WHEAT FLOUR AND WHEAT SOURDOUGH 19

2.7 PROTEIN PROFILES 20

2.7.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis Analysis *20*

2.7.2 Liquid chromatography-mass spectrometry *22*

2.7.3 Competitive R5 ELISA *22*

3 RESULTS **25**

3.1 DETERMINATION OF TOTAL NITROGEN (N) CONTENT IN WHEAT FLOUR AND WHEAT SOURDOUGH 25

3.2 PROTEIN PROFILES OF WHEAT FLOUR AND WHEAT SOURDOUGHS 26

3.3 *IN VITRO* DIGESTION OF THE WHEAT FLOUR AND THE FOUR WHEAT SOURDOUGHS 26

3.3.1 Characterization of human juices, pH analysis and protein profiles *26*

3.3.2 Changes in protein profiles during digestion *30*

3.3.3 Detection of immune reactive amino acid sequences in gluten proteins and peptides by competitive R5 ELISA *33*

4 DISCUSSION **39**

4.1 PROTEIN EXTRACTION FROM WHEAT FLOUR AND WHEAT SOURDOUGH 39

4.2 THE GLUTEN PROTEINS PROFILES OF WHEAT FLOUR AND WHEAT SOURDOUGHS 40

4.3 *IN VITRO* DIGESTION OF WHEAT FLOUR AND WHEAT SOURDOUGHS 41
5 CONCLUSION 45
6 FUTURE PERSPECTIVES 46
7 REFERENCES 47
ATTACHMENT 1: 51

1 Introduction

1.1 Wheat

One of the most important food grains in the world today is wheat. Wheat belongs to the genus Triticum, a member of the grass tribe Triticeac with in the Pooideae subfamily of grasses, and is found in diploid, tetraploid and hexaploid forms. The hexaploid form (Triticum aestivum) is the most common in world agriculture. The wheat grain (86- 89 % dry matter) (Figure 1) consists of two different organs surrounded by the protective bran; the starchy endosperm and the embryo. The endosperm constitutes the biggest part of the mature grain. (Shewry and Halford, 2003). The major component of wheat is carbohydrates, mainly starch (55-70% of dry matter) in the endosperm and fibre (2-13% of dry matter) in the bran. The second largest component is protein (8-11% of dry matters), mainly storage proteins (gluten, 70% of total protein) in the endosperm. Other proteins, such as the metabolic proteins (globulins and albumins) are located in the bran and the germ. The minor components in wheat are fat (2-4% of dry matter) and minerals (1-3% of dry matters) (Koehler and Wieser, 2012).

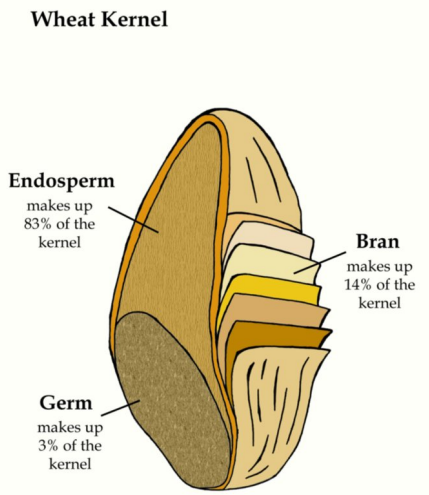


Figure 1: *The wheat grain (kernel)(Ideallustration, 2014)*

Wheat is mainly used as flour in food production and most often consumed after being processed in to bread and other bakery goods, or pasta. The hypersensitive response to wheat in the diet has long been a public health problem, and is one of the most common causes of allergy in the world. The most known hypersensitive reaction to wheat is the autoimmune

disease celiac disease (CD). In addition to CD, wheat allergy gives symptoms of adverse reaction to wheat flour. The component in wheat responsible for these hypersensitive reactions is the wheat protein gluten. Also a non-autoimmune or non- allergenic reaction to gluten is getting more and more common, and is defined as gluten sensitivity (GS) or wheat intolerance (Sapone. et al., 2012).

1.1.1 Gluten

Gluten is a group of proteins that are present either as monomers or as oligo- and polymers linked by interchain disulphide bonds, hydrogen bonds, ionic bonds and hydrophobic bonds. It is the proteins in wheat, and plays a unique role in wheat´s baking quality. The gluten proteins are unique in their amino acid compositions, because of the high content of glutamine and proline, and low content of amino acids with charged side groups. Gluten is divided in to two groups, the alcohol soluble gliadins and the insoluble glutenins (Wieser, 2007).

Gliadins are initially classified into four groups α-, β-,ω-, γ-gliadins, based on their mobility at low pH in gel electrophoresis. α-and β- gliadins have been shown to be very similar, and are referred to as α/β-gliadins. ω-gliadins are characterized by the highest content of proline and glutamine, and most of them lack cysteine and the possibility to make covalent disulphide bonds. The α/β- and γ-gliadins have much lower content of proline and glutamine than ω-gliadin. α/β-gliadin differ from γ-gliadins in the content of tyrosine. The distribution of the different gliadins in wheat varies, but generally the α-,β- and γ-gliadins occur in higher proportions than the ω-gliadins (Wieser, 2007).

Glutenins comprise aggregated proteins linked by interchain disulphide bonds. The dominating group of gluten proteins are the low molecular weight glutenin subunits (LMW-GS). This group is related to the α/β- and γ-gliadins in molecular weight (Figure 2) and amino acid composition. LMW-GS have eight cysteines, six of them are proposed to be linked by intrachain disulphide bonds. The last two cysteines form interchain disulphide bonds with cysteine on other gluten proteins. A smaller group of glutenines are high molecular weight glutenin subunits (HMW-GS), which belong to the minor components of the gluten protein family. The glutenins in this group consist of three structural domains. As they are not present in flour or dough as monomers it is assumed that they form interchain disulphide bonds. A

small part of the glutenins belongs to the largest proteins in nature, they are termed “glutenin macro polymers” and are thought to be one of the determinants of dough properties and baking performance (Wieser, 2007).

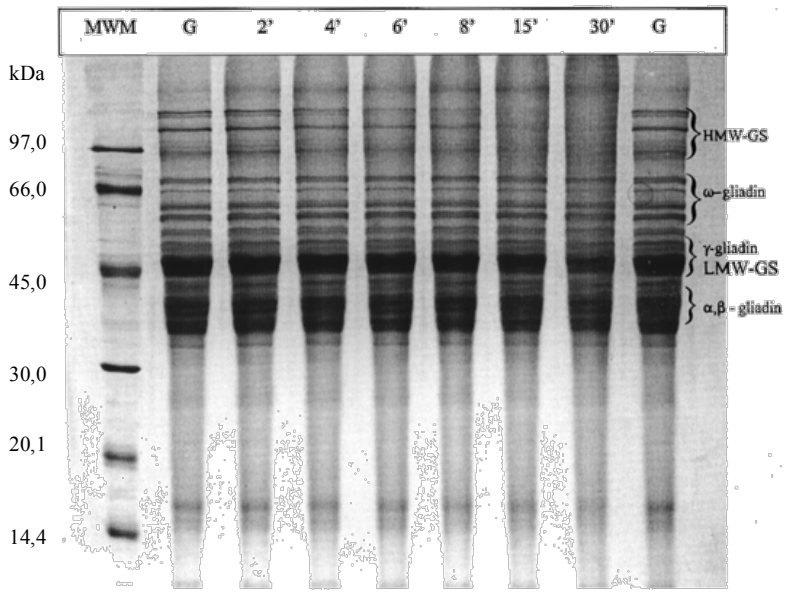


Figure 2: *SDS-PAGE pattern of gluten according to molecular weight (Morel et al., 2002).*

1.1.2 Gluten proteins function in bread

Both the gliadins and glutenins are important contributors to rheological properties of dough. The gliadins contribute mainly to the viscosity and extensibility, while the glutenins are responsible for dough strength and elasticity. A mixture of these two is essential for the viscoelasticity and the quality in the end product (Uthayakumaran. et al., 2000, Wieser, 2007). The amino acid cysteine constitutes a small part of gluten proteins (≈2%), nevertheless it is extremely important for the functionalities of gluten. Most cysteines form either intrachain disulphide bonds within a protein or connect proteins via interchain disulphide bonds -in both cases the cysteines are present in an oxidized state. These bonds are targets for redox reactions during maturing of the kernel, milling, dough preparation and baking (Wieser, 2007). Together with the tyrosine- tyrosine crosslinks between proteins during bread baking and other bonds, the gluten network (Figure 3) is formed that gives the end product good quality (Tilley. et al., 2001, Wieser, 2007). Gluten is also important to get a good crumb structure (Demirkesen. et al., 2010) and keeps the bread airy, because the gluten network has gas holding properties (Gallagher. et al., 2004).

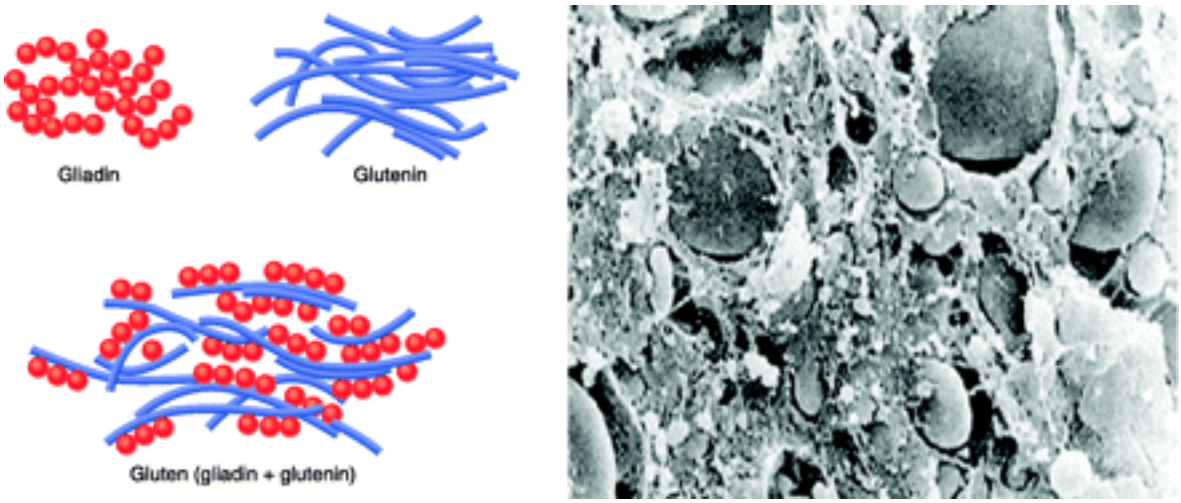


Figure 3: *The gluten network. On the left a cartoon of gliadin and glutenin, and how they interact to form the gluten network. On the right, a scan electron micrograph that shows the structural interaction between gliadins and glutenins (Fasano., 2011).*

Making high quality bread without gluten gives a major technological challenge. The absence of gluten often results in a liquid batter, rather than a dough prebaking, and the consequence is bread with crumbling texture. To get the same texture as gluten-containing bread, the gluten must be replaced. Many gums/hydrocolloids have been tried as gluten replacements, as gums/hydrocolloids have structure-building and water-binding properties and thereby improve the texture of gluten-free bread. Most gluten-free products that are made with refined flour may not contain the same level of nutrients as products containing gluten. Studies done on adults diagnosed with CD have shown a lower intake of dietary fibre compared to a control group on a normal diet. Dietary fibre has long been recognized to contribute to a healthy intestine (Gallagher. et al., 2004). Research has also shown that a gluten-free diet may be low in iron, folate, calcium, magnesium, zinc and B-complex vitamins (Dessi. et al., 2013). So in addition to technological challenges, a gluten- free diet may also lead to lack of nutrients (Gallagher. et al., 2004). Gluten-free products are also more expensive (Arendt. et al., 2011).

1.1.3 Proteins related to wheat gluten

Rye and barley are like wheat also members of the grass tribe Triticeae. The proteins in these two cereal types are similar to gluten in wheat, and are called hordein in barley and secalin in rye. Like wheat they have a high content of glutamine and proline that gives them the same unique processing properties and allergenicity as wheat (Tatham. and Shewry., 2012).

1.2 Celiac disease

Celiac disease (CD) is one of the most common autoimmune diseases (Gujral. et al., 2012). The prevalence is estimated to be 1% of the population (Green and Cellier, 2007, Volta and Villanacci, 2011). It is characterized by the small intestines sensitivity to gluten, which are proline- and glutamine rich proteins in wheat. Barley and rye contain similar proline and glutamine rich proteins, hordeins in barley and secalins in rye. These proteins are thought to be partly resistant to digestion by gastrointestinal proteases, which lead to longer peptides in the small intestine that cause inflammation in CD patients. This inflammation most common leads to mucosal injury and malabsorption (Green and Cellier, 2007, Gujral. et al., 2012, Kagnoff, 2007, Schuppan. et al., 2009). The degree of inflammation varies from patient to patient it can be a plain intraepithelial lymphocytosis or it can infiltrate the mononuclear cells in the sub epithelial layer (lamina propria) and cause total villous atrophy and crypt hyperplasia (Schuppan. et al., 2009).

Celiac Disease is closely connected to genetic factors. That is shown by the presence of specific major histocompatibility complex (MCH) class II HLA-DQ alleles. Almost all biopsy-confirmed CD patients express HLA-DQ alleles that encode specific MCH class II Heterodimers HLA-DQ2 and/or HLA-DQ8 heterodimers on antigen presenting cells (APCs) such as dendritic cells or macrophages that are present in the lamina propria of the small intestine (Kagnoff, 2007, Schuppan. et al., 2009). To be able to get to the lamina propria and the APCs, the toxic gluten, secalin and hordein peptides trigger the innate immune response of the intestine. This generates the release of interleukin-15 (IL15) from the epithelial cells and dendritic cells in lamina propria. IL-15 affects the epithelial barrier by increasing the permeability through disrupting the tight junctions. This disrupting of the tight junctions then allows the undigested allergenic peptides to reach the lamina propria. (Gujral. et al., 2012).

Enzyme tissue transglutaminase (tTG) is also central in celiac disease. The tTG deamidates glutamines in the toxic peptides to hydrophobic residues or crosslink the glutamines to lysine of a second protein (Figure 4). These modifications by tTG give the toxic peptide higher affinity for HLA-DQ2 and HLA-DQ8 (Gujral. et al., 2012, Schuppan. et al., 2009). 31 different peptides have been identified as substrates of tTG, and the majority of these peptides are known toxic epitopes (Dørum. et al., 2010).

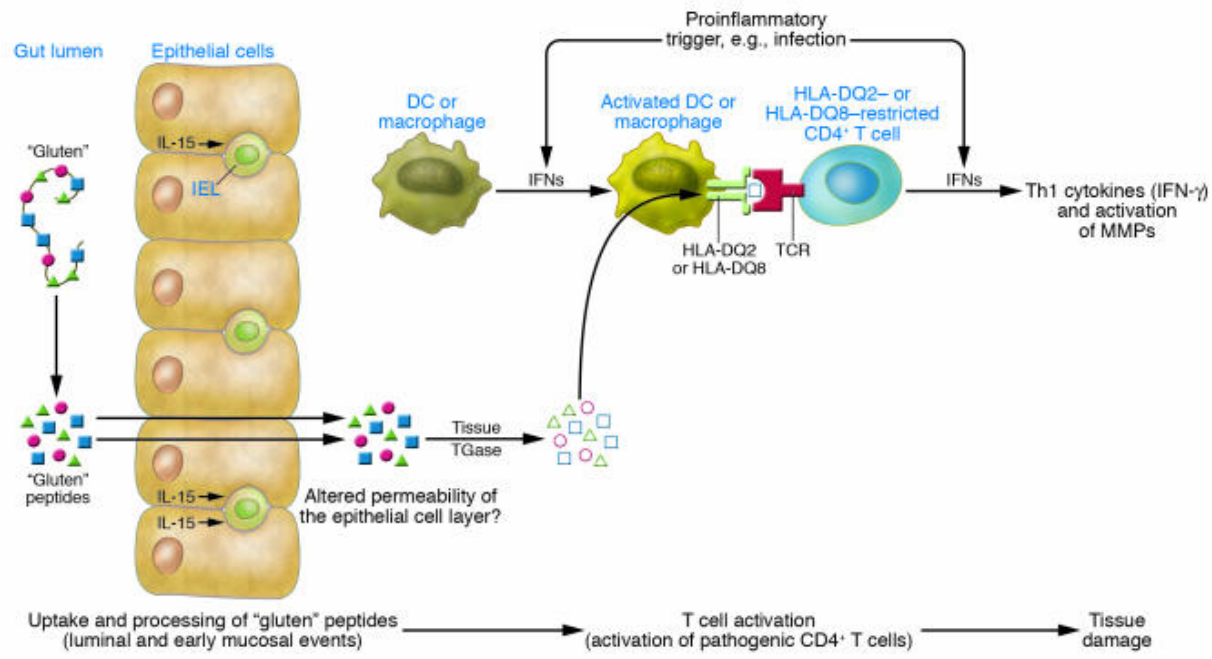


Figure 4: *Pathogenesis of celiac disease (Kagnoff, 2007).*

In lamina propria the toxic peptides bind the HLA-DQ2 and/or HLA-DQ8 on the APCs, which further present them to $CD4^+$ T-helper 1(Th1) cells in the lamina propria (Figure 4). The presented peptide binds the T-cell receptor (TCR)(Gujral. et al., 2012, Kagnoff, 2007, Schuppan. et al., 2009). Larger peptides containing several HLA-DQ2 binding epitopes stimulate CD4+ Th1 cells better than peptides containing a single HLA-DQ2 binding epitope (Kagnoff, 2007). When activated by a toxic peptide, $CD4^+$T-cells produce high levels of pro-inflammatory T-helper1 and T-helper2 cytokines. The T-helper1 cytokines such as INF-γ bind and activate CD8+T-celles and Natural killer (NK) cells. This activation causes apoptotic death of the enterocytes by the Fas/Fasligand system. This causes epithelial damage in the intestine. The T-helper2 (Th2) cytokines such as IL-18, INF-α, or IL21 stimulates the differentiation of B-cells to plasma cells that produce the immunoglobulin´s, anti-gliadin and anti-tTG (Gujral. et al., 2012, Schuppan. et al., 2009). Interaction between anti-tTG antibody and the extracellular tTG is thought to cause epithelial damage (Gujral. et al., 2012)

The production of Th1 and Th2 antibodies promotes more toxic peptides to enter the lamina propria through transcytosis or retrotranscytosis. The transcytosis is trigged by INF-γ, which is a Th1 cytokin. INF-γ drives the transport of toxic peptides from the apical side of the enterocyte cell in the intestine to the basal side and lamina propria (Gujral. et al., 2012, Schuppan. et al., 2009). The retrotranscytosis is driven by CD patients' overexpression of the

transferrin receptor CD71 on the apical side of the enterocyte cells in the intestine. This transferrin CD71 form a secretory immunoglobulin- gliadin complex that secretes the immunoglobulin's anti-gliadin and anti-tTG from lamina propria in to the lumen of the intestine. This secretion allows protected transport of intact toxic peptides in to the lamina propria (Gujral. et al., 2012, Matysiak-Budnik et al., 2008).

IL-15 also acts on intraepithelial lymphocytes (IELs) by up-regulating the NKG2D receptors on natural killer- IELs, and it´s epithelial ligand MICA on enterocytes. MICA functions as a signal on cellular distress and it is normally expressed moderately in villous gut epithelial, but untreated CD patients have a much more intense expression of MICA than normal persons. The MICA/NKG2D interaction directly induces killing of epithelial cells by the IELs, and helps development of villous atrophy (Gujral. et al., 2012, Hue et al., 2004).

1.3 The human digestive system and its enzymes

The human digestive system (Figure 5) is a system of organs that processes food. During this process it absorbs nutrients and eliminates the residue. It is a process that happens in five stages; ingestion, digestion, absorption, compaction and defecation (Saladin, 2012).

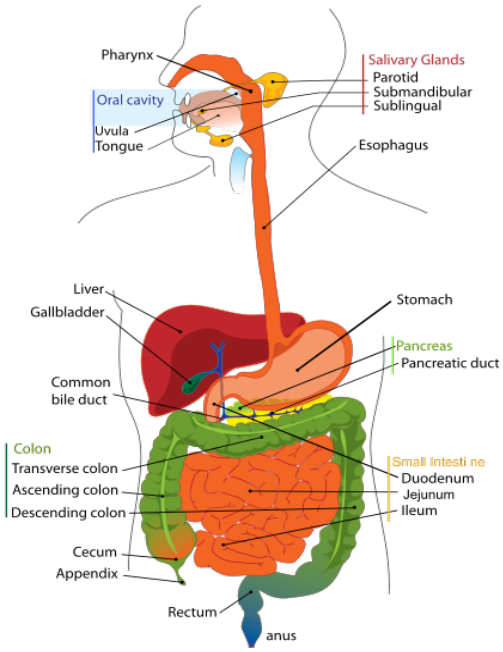


Figure 5: ***Overview over the digestive system*** *(Modric, 2011)*

1.3.1 Mouth, pharynx and esophagus

The mouth has many functions. It is where the food is ingested trough the oral fissure (opening between the lips) and tasted by the taste buds on the tongue. The digesting starts in the mouth with chewing and mixing with saliva, especially the digestions of starch since the saliva contain the enzyme amylase. The enzyme lingual lipase is also secreted in the mouth by lingual glands, but it is pH-dependent and starts the digestion of fat at low pH. In addition the mouth is also the place for speech, and it is a part of the respiration system. When the food is chewed it is swallowed trough the pharynx and down into the esophagus. In the pharynx the digestive tract intersects with the respiratory tract. When food is swallowed the vestibular folds adduct to close the airway and the pharyngeal constrictors force the food downward into the esophagus. The esophagus is a straight muscular tube that leads the food from the pharynx down to the ventricle. The opening into the ventricle is called the cardiac orifice. The lower esophageal sphincter (LES) briefly pauses the food at this opening, before it is lead in to the ventricle (Saladin, 2012).

1.3.2 The ventricle

The ventricle is a j-shaped muscular sac in the upper left abdominal cavity. The main function of the ventricle is storage of food. While storing the ventricle mechanically brakes up food particles and liquefies the food with gastric juice in to a mixture called chyme. The gastric juice is a combination of mainly mucus secreted by the mucous cells, hydrochloric acid (HCl) secreted by the parietal cells, and gastric lipase and pepsin secreted by the chief cells. The two latter starts the chemical digestion of following fat and proteins in the ventricle. The ventricle is divided in to four regions: the cardiac region (cardia), the fundic region (fundus), the body (corpus) and the pyloric region. The latter is subdivided into the antrum and the pyloric channel. The pyloric channel ends at the pylorus, a narrow passage into the duodenum. A thick muscle ring called the pyloric sphincter controls the pylorus passage and the entering of chyme in to duodenum (Saladin, 2012).

Enzymatic digestion of proteins in the ventricle: Digestion of dietary proteins starts in the ventricle, where pepsins starts cleaving peptide bonds between aromatic amino acids such as phenylalanine or tyrosine and a second amino acid, which gives products of peptic digestion polypeptides with varying sizes (Table 1). Pepsins are secreted as inactive proenzymes called pepsinogen from the Chief cells in the deep glands in the gastric mucosa, and activated by gastric acid (HCl). Pepsins have a pH optimum of 1.6 to 3.2, and the activity is terminated

when the chyme is mixed with the alkaline pancreatic juice in duodenum (Barret. et al., 2010, Untersmayr. and Jensen-Jarolim, 2008, Whitcomb and Lowe, 2007).

1.3.3 Small intestine

The small intestine is the longest part of the digestive tract, about 2.7 to 4.5 metres in a living person, and is where almost all digestion and nutrient absorption occurs. It is divided into three parts: the duodenum, the jejunum and the ileum. The duodenum starts where the pylorus passage ends. It has a major and a minor papillae, where it receives alkaline pancreatic juice (pH 8) from the pancreas and bile from the liver. The pancreatic juice contains digestive inactive proenzymes (Table 1) and the bile contains bile salts, which emulsify the fat into smaller droplets, making them easier to digest. When entering the duodenum the pancreas juice mixes with the bile and raises the low pH of chyme to 6.0 - 7.0, which is the optimum pH for the pancreatic enzymes. The proenzymes are activated and together with the other enzymes the chyme is further digested. Most of the digestion and nutrient absorption happens when the mixture is passing down the jejunum, the jejunum has a thick muscular wall and is especially rich in blood supply. The jejunum passes over into the ileum. The ileum has thinner walls and less blood supply than the jejunum, little digestion and absorption happens here. In the end of the ileum is the ileocecal junction where the ileum is connected to the large intestine. Around this transition is a thick layer of muscles that form a sphincter called the ileocecal valve. This sphincter regulates the passage of food residues into the large intestine and prevents feces from backing up into the ileum (Saladin, 2012).

Table 1: *Principal digestive enzymes (Barret. et al., 2010).*

Source	Enzyme	Activator	Substrate	Catalytic Function or Products
Salivary glands	Salivary α-amylase	Chloride	Starch	Hydrolyses 1:4α linkages, producing α-limit dextrins, maltotriose, and maltose
lingual glands	Lingual lipase		Triglycerides	Fatty acids plus 1,2-diacylglycerols
Ventricle/stomach	Pepsins	HCl	Proteins and polypeptides	Cleave peptide bonds adjacent to aromatic amino acids
	Gastric lipase		Triglycerides	Fatty acids and glycerol
Exocrine pancreas	Trypsin	Entero-peptidase	Proteins and polypeptides	Cleave peptide bonds on carboxyl side of basic amino acids (arginine or lysine)
	Chymotryosins	Trypsin	Proteins and polypeptides	Cleave peptide bonds on carboxyl side of aromatic amino acids
	Elastatse	Trypsin	Elastin and some other proteins	Cleaves bonds on carboxyl side of aliphatic amino acids
	Carboxypeptidase A	Trypsin	Proteins and polypeptides	Cleave carboxyl terminal amino acid that have aromatic or branched aliphatic side chains
	Carboxypeptidase B	Trypsin	Proteins and polypeptides	Cleave carboxyl terminal amino acid that have basic side chains
	Colipase	Trypsin	Fat droplets	Facilitates exposure of active site of pancreatic lipase
	Pancreatic lipase		Triglycerides	Monoglycerides and fatty acids
	Bile salt. Acid lipase		Cholesteryl esters	Cholesterol
	Cholesteryl ester hydrolase		Cholesteryl esters	Cholesterol
	Pancreatic α-amylase	Chloride	Starch	Same as salivary α-amylase
	Ribonuklease		RNA	Nucleotides
	Deoxyribonuklease		DNA	Nucleotides
	Phospholipase A^2	Trypsin	Phospholipids	Fatty acids, lysophospholipids
Intestinal mucosa	Enteropeptidase		Trypsinogen	Trypsin
	Aminopeptidases		Polypeptides	Cleave amino terminal amino acid form peptide
	Carboxypeptidases		Polypeptides	Cleave carboxyl terminal amino acid from peptide
	Endopeptidases		Polypeptides	Cleave between residues in midportion of peptide
	Dipeptidases		Dipeptides	Two amino acids
	Maltase		maltose, maltotriose, α-dextrines	Glucose
	Lactase		Lactose	Galactose and glucose
	Sucrase		Sucrose; also maltose and maltotriose	Fructose and glucose

Enzymatic digestion of proteins in the small intestine: The polypeptides formed by the digestion in the ventricle are further digested in the small intestine by proteolytic enzymes from pancreas and the intestinal mucosa. The enzymes aimed for proteins and peptides secreted with the pancreas juice are trypsin, chymotrypsin, carboxypeptidase A, carboxypeptidase B and elastase. Trypsin, chymotrypsin and elastase are endopeptidases, and when activated they act on interior peptide bonds in the polypeptide peptide molecules (Barret. et al., 2010). Trypsin cleaves bonds next to arginine and lysine. Trypsin is also the activator of other inactive pancreatic enzymes (Table 1). Chymotrypsin cleaves the bonds

next to phenylalanine, tryptophan, methionine, tyrosine, asparagine and histidine, and elastase cleaves the polypeptides into smaller polypeptides and tripeptides (Whitney and Rolfes, 2010). Carboxypeptidasene are exopeptidases that hydrolyse the amino acids on the carboxyl ends polypeptides. The last part of the digestion is done by the aminopeptidases, carboxypeptidases, endopeptidases and dipeptidases in the brush- boarder membrane (BBM), and the end products are commonly amino acids, di-, or tripeptides, which are absorbed by the enterocytes and transported over the epithelial layer. There is very little absorption of larger peptides (Barret. et al., 2010, Whitcomb and Lowe, 2007).

1.3.4 The large intestine

The last part of the digestive tract is the large intestine. In the large intestine most of the water and salts are absorbed. It consists of four regions; the cecum, the colon, the rectum and the anal canal. The cecum is the first part after the ileocecal valve, and the appendix is attached to this part. The colon follows the cecum, which is the largest part of the large intestine. It is divided in to the sub regions: ascending colon, transverse colon, descending colon and sigmoid colon. The sigmoid colon connects to the rectum. The rectum has three transverse rectal valves, which enable it to retain feces while passing gas. Stretching of the rectum is what stimulates the defecation reflexes. The last three centrimeters of the digestive tract is the anal canal, which opens out into the anus, the place where the defecation leaves the body (Saladin, 2012).

The large intestine is the home for over 800 species of bacteria, which together are termed the bacterial flora. This bacterial flora is beneficial. It has the ability to digest cellulose, pectin and other plant polysaccharides which humans lack enzymes to digest themselves. Some of the bacteria also synthesize vitamin K and b vitamins. In these ways the bacterial flora help provide nutrients (Saladin, 2012).

1.3.5 Immune reactive gluten peptides

Many immune reactive gluten peptides that are recognized by T-cells are identified from α/β-gliadins, ω-gliadins, γ-gliadins, LMW-glutenins, HMW-glutenins, secalins and hordeins (Table 2). These T-cell epitopes are commonly found within long proline-rich fragments (Sollid. et al., 2012). A 33-mer peptide from α-gliadin (Table 2) contain six overlapping amino acid sequences that can be deamindated by tTG and bind strongly to HLA-DQ2. For

that reason this 33-mer peptide is known as a "super antigen" in celiac patients (Gujral. et al., 2012, Schuppan. et al., 2009).

Table 2: *Immunogenic gluten peptides (Arendt. et al., 2011)*

Amino acid sequences	Position	Immunogenicity
VRVPVPQLQPQNPSQQQPQ	α-gliadin: 1–19	+
QNPSQQQPQEQVPLVQQQ	α-gliadin: 11–28	+
QVPLVQQQQFPGQQQPFPPQ	α-gliadin: 21–40	+
PGQQQPFPPQQPYPQPQPF	α -gliadin: 31–49	+
FPGQQQPFPPQQPYPQPQPF	α-gliadin: 30–49	+
QPYPQPQPFPSQQPYLQL	α -gliadin: 41–58	+
PQPFPSQQPYLQLQPFPQ	α-gliadin: 46–63	+
PQPQLPYPQPQLPY	α-gliadin: 62–75	+/+++
QLQPFPQPQLPY	α -gliadin: 57–68	+/+++
QLQPFPQ	α-gliadin: 57–63	+++
LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (33-mer)	α-gliadin: 57–89	+/+++
QLQPFPQPQLPY	α -gliadin: 58–69/(a)	+/+++
PQPQLPYPQPQLPY	α-gliadin: 63–76/(a)	+/+++
PFRPQQPYPQPQPQ	α-gliadin: 93–106 (a)	+
LIFCMDVVLQ	α-gliadin: 123–132	+
QQPLQQYPLGQGSFRPSQQNPQAQG	α-gliadin: 198–222	+
QYPLGQGSFRPSQQNPQA	α-gliadin: 203–220/(a)	+/+
PSGQGSFQPS	α -gliadin: 205–214	-
PSGQGSFQPSQQ	α -gliadin: 205–216/(a)	+/+++
SGQGSFQPSQQN	α –gliadin: 206–217/(a)	+/+++
QGSFQPSQQN	α-gliadin: 208–217/(a)	-/+++
LQPQQPFPQQPQQPYPQQPQ	γ-gliadin: 60–79	+
FPQQPQQPYPQQPQ	γ -gliadin: 66–78	+
FSQPQQQFPQPQ	γ -gliadin: 102–113	-/+
OQPQQSFPEQQ	γ -gliadin: 134–153/(a)	+/+++
VQGQGIIQPQQPAQL	γ-gliadin: 222–236/(a)	+/+
QQQQPPFSQQQQSPFSQQQQ	glutenin: 40–59/(a)	-/+
QQPPFSQQQQPLPQ	glutenin: 46–60/(a)	-/+
SGQGQRPGQWLQPGQGQQGYYPTSPQQSGQGQQLGQ	glutenin:707–742/(a)	+/+
PGQGQQGYYPTSPQQSGQ	glutenin: 719–736	+
GYYPTSPQQSGQGQQLGQ	glutenin: 725–742	+
GYYPTSPQQSG	glutenin: 725–735	+
QGYYPTSPQQS	glutenin: 724–734/(a)	-+
QQGYYPTSPQQSG	glutenin: 723–735	+
GQQGYYPTSPQQSG	glutenin: 722–735	+
GQQGYYPTSPQQS	glutenin: 722–734	+

(a): deamidated

1.3.6 Digestion of gluten

Generally oligopeptides are efficiently hydrolysed into amino acids, di, -or tripeptides by peptidases in the brush border membrane after the proteolytic activity of pancreatic proteases (Stepniak. et al., 2006). Hausch et al. did a digestion trial of a washed allergenic gluten peptide, PQPQLPYPQPQLPY (Table 2) with the pancreatic enzymes trypsin, chymotrypsin, elastase and carboxypeptidases from rats. After digestion with elastase for 75 minutes, the

peptide was partly digested PQPQLPYPQPQ↓LPY. Further digestion of these fragments for another 75 minutes with trypsin and chymotrypsin did not show any further proteolytic fragments. That was expected since the peptide does not contain any trypsin- or chymotrypsin- cleaving sites. The exopeptidase carboxypeptidase A was capable of completely releasing the COOH- terminal tyrosine of PQPQLPYPQPQLPY, but the resulting COOH-terminal proline residue blocked further proteolysis (Hausch. et al., 2002). After *in vitro* digestion preformed by Comino et al. (2012) using commercial enzymes, the results showed that the 33-mer peptide known as "super antigen" remained intact (Comino et al., 2012). *In vitro* digestion done by Shan et al. (2002) with brush border membrane preparations from rat intestine showed that after digestion for 1 to 5 hours the control peptides were nearly completely proteolysed, but the 33-mer peptide remained largely intact under digestion for at least 15 hours. *In vivo* studies done on rats gave the same result, indicating that the 33-mer peptide is very stable when exposed to the brush border membrane of the upper small intestine. *In vitro* digestion with human brush border membrane biopsy preparation from five individuals gave the same results as the *in vitro* digestion with brush border membrane preparation from rat. (Shan. et al., 2002). The high content of proline in gliadins, glutenins, hordeins and secalins is thought to make these proteins resistant to complete proteolytic digestion by the gastric, pancreatic and brush border membrane enzymes in the human intestine (Hausch. et al., 2002, Kagnoff, 2007, Schuppan. et al., 2009). This feature can most likely be connected to disease-inducing properties of gluten (Stepniak. et al., 2006). This can result in accumulation of peptide fragments as long as 50 amino acids with high proline and glutamine content, and T-cell epitopes inducing celiac disease (Hausch. et al., 2002, Kagnoff, 2007).

1.4 Sourdoughs

Sourdough is used in bread and other bakery goods mostly to improve flavour, volume and shelf-life (Gocmen et al., 2007, K.Katina. et al., 2006). To make sourdough old fermentation method is used. Fermentation of cereal is one of the oldest biotechnological processes- it was used in ancient Egypt, where both bread and beer were made with help from lactic acid bacteria (LAB) and yeast (Poutanen. et al., 2009). A sourdough is made with a microbial starter culture of LAB and yeast (ratio 100:1) that are added to a mix of flour and water. The mixture is then allowed to ferment at 25-30°C, usually overnight exposed to the atmosphere. More flour and water is then added together with salt and fat, and the dough is set to ferment

for a short time before it is ready to be used in baking (Salim-ur-Rehman. et al., 2006). The synergistic effect between the LAB and the yeast is important for the metabolic activity of the sourdough. During the fermentation time the LAB produce lactic and acetic acids and thus lower the pH, usually below 5.0. The yeast produces carbon dioxide and ethanol. These changing conditions in the sourdough during fermentation contribute to activation of the cellbound enzymes in the LAB. Especially the change in pH enhances the performance of certain enzymes such as proteases. The enhanced activity of these enzymes, together with the microbial metabolites, is what gives the technological and nutritional effects of fermented cereal foods (Arendt. et al., 2011, Poutanen. et al., 2009). This activation of enzymes has in recent years led to research on sourdough´s ability to hydrolyse the toxic gluten peptides. The reported data suggest that with long-time fermentation and presence of specific LAB, sourdough seems to reduce the amount immune reactive gluten peptides (Cagno. et al., 2004, Caputo. et al., 2010).

1.5 Treatment of celiac disease

The only effective treatment of celiac disease today is a strict lifelong gluten- and related protein- free diet. In reality this is not as easy as it sounds, due to the gluten from food contamination (Gujral. et al., 2012). A gluten-free diet is also demanding and hard to maintain because of social isolation, financial issues or restriction in food diversity (Pinier et al., 2010). Modifications to dietary gluten have been tried to make it non-toxic. This has not been a success because the glutens properties in bakery products have been lost (Gujral. et al., 2012). To recognize the products tolerated by celiac patients, the Norwegian food authority (Mattilsynet) has set the gluten protein limit <100 mg/kg to label a product *low in gluten*, and the limit <20 mg/kg to label it *gluten-free (Helse-ogOmsorgsdepartementet, 2009)*.

1.6 Aim of study

The aim of this study was

- To detect differences in the gluten protein profile of wheat flour and wheat sourdoughs with different starter cultures
- To digest gluten in wheat flour and selected wheat sourdoughs and detect immunogenic amino acid sequences of gluten proteins and peptides after *in vitro* digestion using human gastrointestinal enzymes from normal persons and celiac patients

2 Materials and methods

2.1 Materials

2.1.1 Wheat flour

The wheat flour used in this study was baker's wheat flour delivered from Regal wheat flour bakery, Stockholm. It contained 12% protein, 68% carbohydrates and 2% fat. Dry matter was 90 % and 10 % water.

2.1.2 Wheat sourdoughs

The four different wheat sourdoughs used in this study were prepared and delivered by Idun Industri AS, Postboks 144, 2026 Skjetten. They were all based on the same batch of wheat flour (Regal wheat flour bakery) and each sourdough was made with its own starter culture. Wheat sourdough 1 contained *Lactobacillus brevis*, Wheat sourdough 2 had *Lauconostoc dextranicum*, wheat sourdough 3 was made with *Lactobacillus brevis* and *Leuconostoc dextranicum*, and Wheat sourdough 4 was made with *Lactobacillus plantarum*. The sourdoughs consisted of 44 % dry matter and 56 % water.

2.1.3 Human Gastric and duodenal juice

Collection of human gastric juice (HGJ) and human duodenal juice (HDJ) was done according to Holm et.al (Holm. et al., 1988) from a batch of 5 normal persons and from two individual celiac disease (CD) patients. HGJ from one celiac disease patient (CD2) and HDJ from another celiac disease patient (CD1) were used because of the large volumes needed for digestion.

2.2 Gluten extraction procedures

To extract gluten from the wheat flour and wheat sourdough two extraction procedures were performed, 1) 60% ethanol extraction and 2) universal prolamin and glutelin extractant solution (UPEX) extraction. Method 2) extracted more gluten than method 1, and was further used as standard extraction procedure.

2.2.1 Ethanol (60%) extraction method

This extraction procedure is based on 60% (vol/vol) aqueous ethanol. Aliquots of 0.25g wheat flour and 0.25g wheat sourdough were weighed into each propylene tube. 10ml of 60% (vol/vol) aqueous ethanol was added to each of the propylene tubes. The tubes were incubated for 1h at room temperature in a rotary shaker (Multi RS-60, BIOSAN) at 45 turns/minute. They were then centrifuged (Heraus Megafuge 1.0) at 2500-g for 10 minutes at room temperature. The supernatants were transferred into two new polypropylene tubes. The extracts were then ready for analysis (García. et al., 2005).

2.2.2 UPEX extraction method

The UPEX extraction procedure is based on reducing Tris (2-carboxyethyl)-phosphine (TCEP) (Sigma- Aldrich) which reduces disulphide bridges, and anionic surfactant N-lauroylsarcosine (Sigma- Aldrich) that contributes to the opening of polypeptide chains. The reagents were diluted in 2,5ml phosphate buffer saline (PBS). 0,25g wheat flour and 0,25g wheat sourdough were weighed in their own propylene tube. 2,5ml of the UPEX solution (5mM TCEP, 2% N-lauroylsarcosine in PBS, pH 7) was added to each of the propylene tubes. The UPEX solution was made immediately before use to prevent inactivation of the reducing TCEP. The tubes were closed tightly and to avoid evaporation the cap was covered with sealing film (Nescofilm). The samples and the UPEX solution were mixed thoroughly by vortexing until the samples were totally dissolved. The tubes were incubated in a water bath (AQUAline AL 18) at 50°C for 40 min. After incubation the tubes were allowed to cool for 5 min in room temperature before 7,5ml 80% (vol/vol) aqueous ethanol was added to each of them. The content in the tubes were again mixed thoroughly by vortexing until the sample was totally dissolved. The tubes were incubated for 1hour at room temperature in a rotary shaker (Multi RS-60, BIOSAN) at 45turns/min. The tubes were centrifuged at 2500g for 10min at room temperature. The supernatant were transferred to polypropylene tubes. The extracts were then ready for analysis (Mena. et al., 2012).

2.3 Measuring protein content

2.3.1 Kjeldahl method

The protein content in wheat flour, wheat sourdough and the different extractions were measured by the Kjeldahl method. The Kjeldahl method is based on analysis of the total

amount organic nitrogen (N) and is much used to measure the protein content in different commodities and foodstuffs. The amount N is recalculated to protein content using a numerical factor, which differs between products. For wheat the factor is 5,7. To measure the amount organic N, the Kjeldahl apparatus Kjeltec^{TM} 8400 (FOSS) was used.

Preparation: To prepare for the Kjeldahl method 500 mg of each sample were weighed in tubes, all samples in three parallels. 3ml concentrated sulphuric acid and a catalyst (in the form of a Kjeldahl tablet) was added to all tubes, including two blind samples. The sulphuric acid breaks down organic N-compounds to ammonium sulphate ($(NH_4)_2SO_4$) under heating, while the catalyst (potassium sulphate) helps raise the boiling point so that the boiling time is reduced. The tubes were positioned in a rack and placed on a heating block. The samples were heated to 420°C and boiled for 45-60 min. When the samples were clear and a condensation ring appeared ¼ from the top of the tube, the tubes were removed from the heating block and chilled (Eijsink. et al., 2013).

Measurement of total N: The tubes were then one by one placed in the Kjeltec^{TM} 8400. In the Kjeltec^{TM} 8400 water and 33% sodium hydroxide (NaOH) were automatically added to the ammonium sulphate-sulphuric acid. This led to the formation of ammonia (NH_3).
The amount of NH_3 is equivalent to the amount organic N. The NH_3 is volatile so after the formation of NH_3, the NH_3 was automatically distilled over a 200ml Erlenmeyer flask, which contained boric acid solution with an added indicator (bromine cresol green). Ammonium (NH_4^+) and Borat ions ($H_2BO_3^-$) are formed. The amount of $H_2BO_3^-$ is equivalent to the amount of NH_3. The amount of $H_2BO_3^-$ is determined using titration with standardised hydrochloric acid (0,05M HCl). The amount of HCl is used to calculate the N content (Eijsink. et al., 2013).

2.4 pH in the human gastric juices and the human duodenal juices

Before digestion the pH was measured with an electrode pH meter (827 pH lab, Metrohm) in the batch of HGJ and HDJ from normal persons, and in HGJ and HDJ from both CD1 and CD2.

2.5 Proteolytic Activities of Gastric and Duodenal Enzymes

Performed by Irene Comi/Ellen Ulleberg.

The pepsin activity of HGJ from normal persons and CD2 was analysed with haemoglobin (Sigma, St. Louis, MO, USA) as substrate, at pH 3. The total proteolytic activity of the HDJ from normal persons and CD1 was analysed at pH 8, using casein (Merck CO., Darmstadat, Germany) as a substrate. To measure the activities three concentrations of HGJ or HDJ in triplicates were incubated with substrate for 10 minutes at 37°C. The reactions were stopped adding trichloroacetic acid. The samples were kept for overnight sedimentation at 4°C, before centrifugation for 10 minutes at 3000×g. The absorbance (A) was then measured at 280nm in a spectrophotometer. One unit of enzyme activity was defined as the amount (ml) of HGJ or HDJ giving a difference in absorbance of 1.0 at A_{280nm} in 10 minutes at 37°C (Ulleberg et al., 2011).

2.6 *In vitro* digestion of wheat flour and wheat sourdough

The wheat flour and all four wheat sourdoughs were digested *in vitro* with 5ml HGJ (26,65 U/ml) and 5ml HDJ (12,4 U/ml) from normal persons, and the wheat flour and wheat sourdough 3 were digested *in vitro* with 5ml HGJ (0,0 U/ml) from CD2 and 5ml HDJ (21,8U/ml) from CD1.

Preparation: Aliquots of 0,250mg sample were weighed into four tubes and diluted with 5ml of distilled water (dH_2O). The tubes were added magnets and placed in a water bath (Julabo) at 37°C with a magnet stirrer (RCT basic, Kika labortechnik). 5ml of HGJ was added to each tube. To adjust the pH to pH 2, pH 4 and pH 7 during the digestion procedure 1M HCl, 1M NaOH and 4M NaOH were used.

Digestion with human juices normal persons: The digestion was performed in two steps, during the gastric step the pH were adjusted to pH 2 (original HGJ was pH 2,06) and to pH 4. The tubes were incubated in the water bath at 37°C under constant stirring for 1 hour. After gastric digestion two tubes, one with pH 2 and one with pH 4 were redrawn and placed on ice to stop proteolysis. The second step of digestion, continued for HDJ digestion by adjusting pH to 7 and adding 5 ml of HDJ to each tube. The tubes were incubated in the water bath at 37°C for 1 hour with constant stirring, and then placed on ice.

Digestion with human juices from CD patients: Under digestion with juices from two different CD patients the pH was unadjusted (original pH 7,4) in two tubes and adjusted to pH 2 in two other tubes. The four tubes were incubated in the water bath at 37°C under constant stirring for 1 hour. Then two tubes were redrawn, one with the pH unadjusted and one with the pH adjusted to pH2 and placed on ice to stop the reaction. In the remaining tube with pH unadjusted 5 ml of HDJ (original pH 7,5) was added. In the other tube adjusted to pH 2, the pH was adjusted to pH 7 and 5ml of HDJ was added. The tubes were incubated in the water bath at 37°C for 1 hour under constant stirring. All digestion samples, from normal persons and CD patients were frozen down before analysis.

2.7 Protein profiles

The protein profile of the wheat flour and four wheat sourdoughs, and proteins profile after the digestion with HGJ an HDJ were analysed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Some of the protein bands were cut out of the gel, and analysed with liquid chromatography-mass spectrometry (LC-MS) The content mg/kg of immune reactive gluten sequences were analysed with competitive R5 ELISA.

2.7.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis Analysis

SDS-PAGE is used to separate proteins according to their change and MW and thus gives a qualitative analysis of protein mixes. It is based on SDS, which is a strong anionic detergent. Not all proteins have the same charge, so mixing them with SDS denatures and charge them negatively. This allows the proteins to separate based on molecular weight. To promote denaturation a reducing agent is added to break disulphide bonds and the samples are boiled, before applying them to the polyacrylamide gel. When voltage is applied to the gel, the negatively charged proteins migrate through the gel at different speeds. Small proteins migrate faster than bigger proteins through the gel network. This results in proteins separating into bands (Jensen, 2012). The SDS-PAGE was used to study change in the protein profile after the different phases of the *in vitro* digestion, and the wheat flour and the four wheat sourdoughs were applied as point zero.

Sample preparation of wheat flour and the wheat sourdoughs: The first step in the preparation was to wash away the starch from the wheat flour and the wheat sourdoughs. That

was done according to Darlington et al. (2000). 0,25g sample was diluted in 5ml of dH_2O and mixed until the sample was totally dissolved and centrifuged at 5000g for 3 min and the supernatant was discarded. The washing was repeated 15 times (Darlington et al., 2000).

Electrophoresis: The rest of the preparation was done using the model from Morel et al. (2002). All the digestion samples and the washed samples were diluted 1:1 with 2x SDS-PAGE sample buffer (0,125M Tris-HCl, 4%SDS, 20%glycerol, 2%Dithiothreitol (DTT), pH 6,8, 10ml) and the standard consisting of phosphorylase b (95kDa), bovine serum albumin (65kDa), ovalbumin (45kDa), carbonic anhydrase (30kDa), soybean trypsin inhibitor (20,1kDa) and lactalbumin (14,4kDa) (Bio-Rad) was diluted with 2x SDS-PAGE sample buffer according to Bio Rad. The samples were held in a water bath for 1 hour at 50°C and then boiled for 5 minutes on a heating block before added to the wells (approximately 45μg gluten proteins to each well in the wheat flour, wheat sourdoughs and digested samples, and 45μg proteins from the digestion juices in the samples of HGJ and HDJ) in the gel placed in the gel comb. The gels used were 12% precast polyacrylamide gel (12% mini-PROTEAN®TGX™) with 10 wells, 30μl each, delivered by Bio-Rad. The gels were covered with 10x Electrode running buffer (1x 0,025M Tris, 0,192M glycine, 0,1%SDS, pH 8,3) diluted 1:10 in dH_2O and the electrophoresis ran for 35 minutes at constant voltage 200V. The electrophoresis was done using BIO-RAD Powerpac basic (BIO-RAD). The electrophoresis of the gel with wheat flour and wheat sourdoughs were run 9 times, while the gels with digestion juices and digested samples were run 3-5 times.

Staining and preservation: After the electrophoresis the gels were put in plastic boxes and fixing solution (20%MeOH in dH_2O) was added for 10 minutes. The fixing solution was removed, and staining solution (0,1% Coomassie R-250, 40% MeOH (95%), 10% HAc in dH_2O) was added, and the gels were stained for 15 minutes. Then the staining solution was removed, and distaining solution (10% HAc,10% MeoH (95%), 80% dH_2O) was added. The distain solution was removed after 15 minutes, and new distain solution was added. This was repeated until the SDS-PAGE protein pattern was clear. The gels where then put in preservation solution (10%glycerol, 10%MeOH (95%), 80%dH_2O) and scanned with perfection U750 pro scanner (EPSON).

2.7.2 Liquid chromatography-mass spectrometry

Performed by Morten Skaugen.

The liquid chromatography-mass spectrometry (LC-MS) method is based on ionizing the peptides and measuring their mass by following their specific paths in vacuum. It can be used to identify proteins from a biological source, were for example the last step in protein purification is SDS-PAGE. In this study bands from the protein profile of the HGJ from celiac patient 1 and celiac patient two were analysed. The band of the unknown protein in a gel was cut in to several small slices and the proteins in these slices were then further in-gel digested using different enzymes and chemicals. Then the peptides were desalted and concentrated before loaded on to a microscale capillary high-performance liquid chromatography (HPLC) column, which was directly connected to the mass spectrometer. At the end of the capillary columns peptides were ionized by electrospray ionization. The ionization of the peptides changed their electrical charge. The charged peptides were then eluted in as small volumes as possible into the mass spectrum, using a solvent gradient of increasing organic content, which made the peptide ions elute in order of their hydrophobicity. The mass spectrometer was a vacuum system. When the peptide ions entered this system they were guided and manipulated by electric fields, and the mass-to-charge (m/z) ratio of the charged particles were determined using an m/z scale. After all the m/z values were determined, the mass spectrometer proceeded to obtain the primary structure of the peptides. The obtained peptide-sequencing data from the mass spectra were searched against protein databases, using a database-searching programme (Steen and Mann, 2004).

2.7.3 Competitive R5 ELISA

The term ELISA is commonly used as a description of all forms of enzyme immunoassay with colorimetric detection principle. An immunoassay is a test used to detect and determine antigen-antibody interaction. Enzyme immunoassays are assays where enzymes are bound to an antibody or an antigen, which makes them possible to detect. The assays are usually heterogenic, which means that antibody or antigen is coated on a microtiter plate and form the attached phase. Competitive ELISA is an enzyme immunoassay based on competition between marked and unmarked antigens, where a competition for binding to an antibody of the attached phase occure (Lea, 2002). A monoclonal R5 antibody was used in the competitive R5 ELISA. R5 marks the pentapeptide that interacts with the conesus sequence of five amino acids glutamine-X-proline-phenylalanine/Tryptophan-proline (Q-X-P-F/W-P).

Variations of this sequence may occur in the celiac-toxic T-helper cell motifs in wheat, barley and rye (Valdés. et al., 2003). The competitive R5 ELISA was done according to Mena. et al. (2012), using a kit (RIDASCREEN Gliadin competitive, R7021) delivered from r-biopharm.

Preparation: To perform the competitive R5 ELISA 250 mg sample was extracted using the UPEX- extraction procedure. Each extraction sample was diluted in three dilutions concentrations; 1:25, 1:50 and 1:100, while the flour and sourdoughs extracts were also diluted 1:400 with a sample diluent enclosed the kit.

Competitive R5 ELISA: The wells in the microtiter plate (a 96 well plate) were coated with gliadin. 50µl of sample was added to each well in the microtiter plate, each in duplicates. Gliadin standards ready-to-use enclosed in the kit (0,00 ng/ml, 10,00 ng/ml, 30,00 ng/ml, 90,00 ng/ml and 270 ng/ml) were also added in 50µl to each well. Then 50µl of peroxidase conjugates R5 antibody was added. The microtiter plate was carefully shaken and incubated at room temperature for 30 minutes. During the incubation the attached gliadin, and the gliadin in the samples competed for the binding site on the R5 antibody. The liquid was then removed from all the wells and the plate was tapped three times against absorbent paper. 250µl diluted washing buffer enclosed in the kit was added to each well and removed, this was repeated two more times. Any R5 antibodies not bound to the gliadin in the attached phase where then washed away. Then 100µl of a chromogen substrate following the kit was added to each well. The mictotiter plate was carefully shaken and incubated for 10 minutes at room temperature. During this incubation the enzymes on the conjugated R5 antibodies converted the chromogen substrate in to a blue product. The amount blue product is proportional to the concentration of the gluten peptides in the sample. A stop reagent of 100µl was added and the absorbance (A_{450nm}) was measured at 450nm within 10 minutes using the Sunrise microplate reader (TECAN). The A_{450nm} was then converted to absorbance % by using the A_{450nm} for 100% absorption, which was 2,34. Using the absorption % the amount gluten peptides (ng/ml) were read from the standard curve following the kit (Figure 11). The amount were then further multiplied by the dilution factor and then recalculated to mg/kg.

A schematic summarize of the entire experiment done in this study is shown in Figure 6.

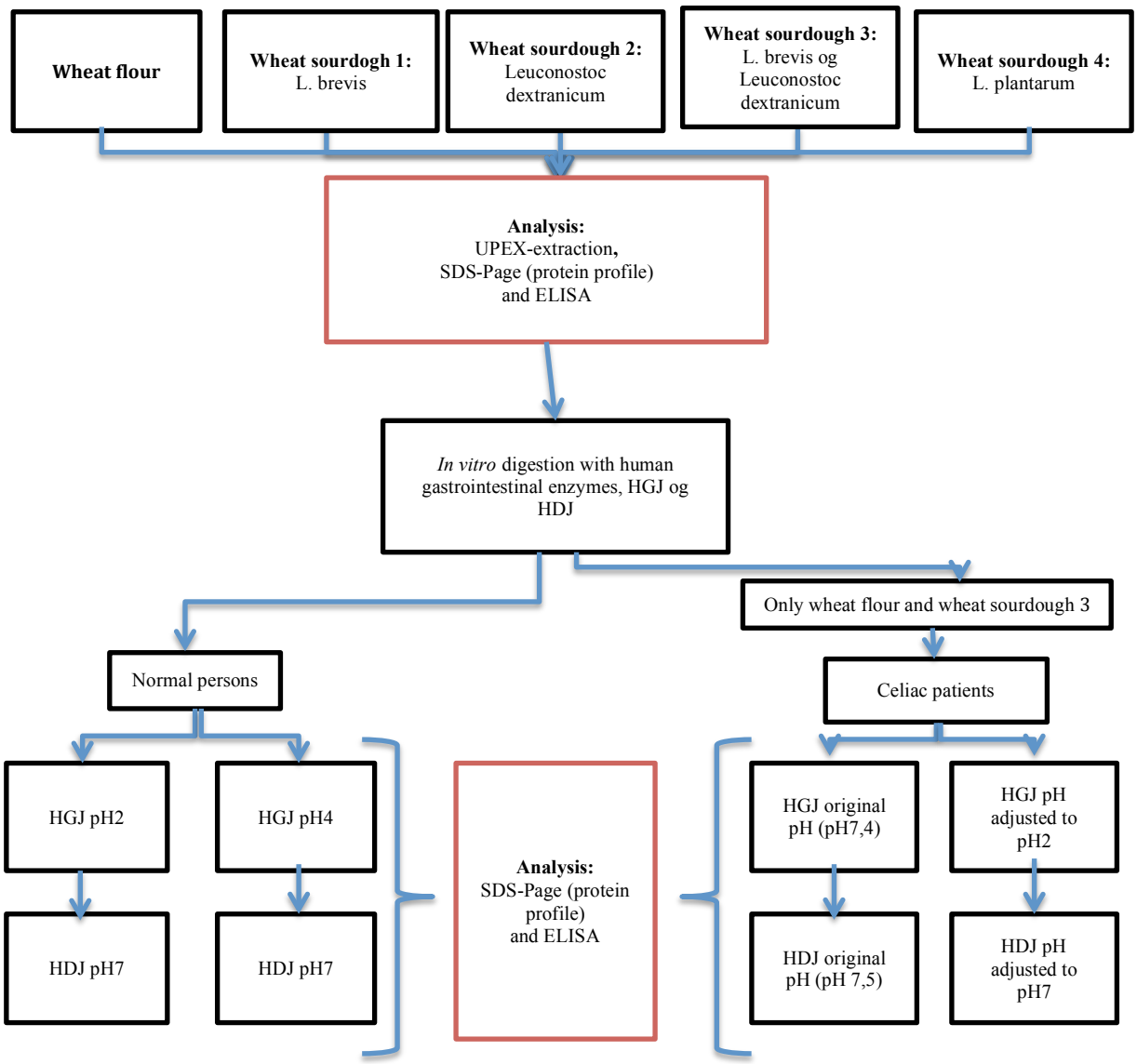


Figure 6: *An summarize of the entire experiment, showing the starting samples; wheat flour, wheat sourdough 1, wheat sourdough 2, wheat sourdough 3 and wheat sourdough 4, and the UPEX, extraction and the analysis (SDS-PAGE and ELISA). Then the in vitro digestion with gastric juice (HGJ) and duodenal juice (HDJ) from normal persons and from celiac patients, and the protein analysis performed; SDS-PAGE and ELISA.*

3 Results

3.1 Determination of total nitrogen (N) content in wheat flour and wheat sourdough

The total nitrogen (N) content of wheat flour and the wheat sourdough was 0,02 mg N/mg dry matter in both samples (Table 3).

Table 3: *Results of the content of N/mg solids in the wheat flour and wheat sourdough measured by the Kjelldahl method.*

	Amount N/mg dry matter
Flour	0,02mg N/mg dry matter
Sourdough	0,02mg N/mg dry matter

The N content in the 60% ethanol extracts and the UPEX- extracts of the wheat flour and wheat sourdough was determined and shown in Table 4. The ethanol extraction (60%) of the wheat flour contained 0,078 mg N/ml. The UPEX-extract of the wheat flour contained 0,19 mg N/ml. Theoretical calculation of the maximum N value of wheat flour and sourdough gave 0,45 mg N/ml and 0,22mg N/ml, respectively. Using ethanol extraction (60%) of sourdough was not possible, due to a very viscous sample. Whereas the UPEX-extraction was able to extract 0,11 mg N/ml. The UPEX-extraction method was the better of the two methods, and it was able to extract 42,2% of the total amount N possible to extract from the wheat flour and 50% possible to extract from the wheat sourdough, as compared to t 17,3% and 0 % extracted with the 60% ethanol extraction method. As the UPEX-extraction method was the better of the two methods, therefor it was used to extract the gluten proteins and peptides to perform the competitive R5 ELISA. The UPEX-extracted digestion samples diluted in gastric juice and duodenal juice had a more clear solution and a smaller pellet after centrifugation, compared to the extractions of undigested wheat flour and wheat sourdoughs.

Table 4: *Content of N/ml extracted from the wheat flour and the wheat sourdough with the 60% ethanol extraction method and UPEX-extraction method compared to the theoretical amount that can be extracted.*

	Theoretical N that can be extracted mg N/ml	60% ethanol extraction method mg N/ml	UPEX-extraction method mg N/ml
Wheat flour	0,45	0,078	0,19
Wheat sourdough	0,22	0,0	0,116

3.2 Protein profiles of wheat flour and wheat sourdoughs

The SDS-PAGE protein profile of the wheat flour compared to the wheat sourdoughs showed that it might be one to two protein bands that seemed to be missing in the sourdoughs (Figure 7, blue arrows). However, it was difficult to compare the individual protein bands due to many bands and differences in the Coomassie blue colouring.

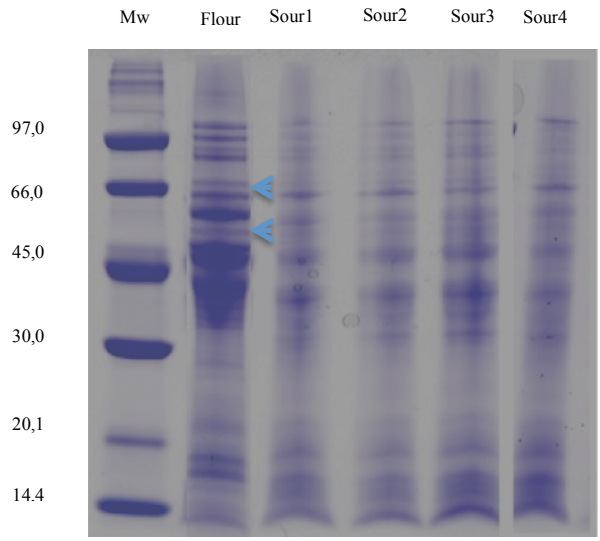


Figure 7: *SDS- PAGE protein profile of the flour and the fours sourdoughs undigested*

3.3 *In vitro* digestion of the wheat flour and the four wheat sourdoughs

3.3.1 Characterization of human juices, pH analysis and protein profiles

Before digestion the pH in the gastric juice from normal persons was measured to pH 2,06 and the duodenal juice to pH 7,1. In the gastric juice and duodenal juice from celiac patient 1 the pH was measured to pH 7,6 in the gastric juice and pH 7,5 in the duodenal juice, and from celiac patient 2 the pH was measured to pH 7,4 in the gastric juice. The proteolytic activity of the gastro intestinal enzymes used for digestion was also measured. The gastric juice from

normal persons had a pepsin activity of 25,65 U/ml and the duodenal juice from normal persons had a total proteolytic activity of 12,4 U/ml. The gastric juice from celiac patient 2 had a pepsin activity of 0,0 U/ml and the duodenal juice from celiac patient 1 had a total proteolytic activity of 21,8 U/ml (Table 5).

Table 5: *pH in the gastric juice (HGJ) and duodenal juice (HDJ) from the normal persons and the CD patients, and the proteolytic activity (U/ml) in the HGJ and HDJ used for digestion.*

	HGJ	**HDJ**
Batch of normal persons	pH 2,06/ 25,65 U/ml	pH 7,1/ 12,4 U/ml
Celiac patient 1	pH 7,6	pH 7,5/ 21,8 U/ml
Celiac patient 2	pH 7,4/ 0,0 U/ml	…*

*The amount was not homogenous, and the pH was not possible to detect.

The protein profile of the gastric juice showed large difference between the gastric juice from normal persons, celiac patient 1 and celiac patient 2 (Figure 8). The gastric juice profile from normal persons contained few protein bands compared to the gastric juice from celiac patient 1, while the gastric juice from celiac patient 2 contained some extra bands compared to the gastric juice from normal persons and much less bands than celiac patient 1. The protein profile of duodenal juice from normal persons and celiac patient 1 seemed more alike, while the profile of duodenal juice from celiac patient 2 showed few enzyme bands. The large number of bands observed in the gastric juice of celiac patient 1 and 2 was further analysed by liquid chromatography-mass spectrometry (LC-MS). The proteins identified are showed in Table 6, were they are compared to proteins identified in HGJ from normal persons from previous study (Ellen Ulleberg, unpublished results). Proteins as Serum albumin and α-amylase indicated that the HGJ from celiac patient 1 and 2 contained blood and refluxed duodenal juice respectively.

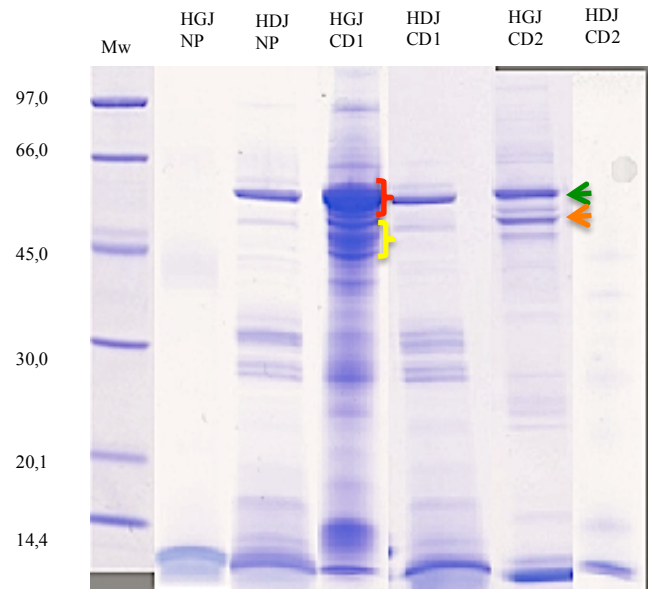


Figure 8: *SDS-Page protein profile of the HGJ and the HDJ from normal persons (NP), HGJ and HDJ from CD patient 1 (CD1) and HGJ and HDJ from CD patient 2 (CD2). The red and the yellow marks the two bands cut out for LC-MS from HGJ CD1 and the green and the orange marks the bands cut out for LC-MS from HGJ from CD2.*

Table 6: *Proteins identified by LC-MS with 100% certainty in the gastric juice (HGJ) from celiac patient 1 and celiac patient 2 (the colour marks behind show which protein band (SDS-PAGE gel) the proteins belonged to). The proteins identified were compared to proteins identified in HGJ from normal persons, detected on an earlier occasion.*

Proteins detected in HGJ form normal persons	Proteins detected in HGJ from Celiac patient 1 and 2
Gastric proteins	**Gastric proteins**
Pepsin A-1	Gastric triacylglycerol lipase (45 kDa) x x
Pepsin III-3	Gastric intrinsic factor (45 kDa) x
Pepsin precursor	**Duodenal proteins**
	α-amylase (58 kDa) x x x x
	α-amylase 2B (58 kDa) x x
	bile salt-activated lipase precursor (80 kDa) x
	Carboxypeptidase E precursor (64 kDa) x x
	Chymotrypsin-like elastase family member 2A (29 kDa) x x
	Chymotrypsin-like elastase family member 3b (29 kDa) x
	Dipeptidyl peptidase 1 (52 kDa) x
	Pancreatic triacylglycerol lipase (51 kDa) x
	Pepsin A preproprotein (42 kDa) x
	Trypsin-1 (27 kDa) x x x x
	Blood proteins
	α1B-glycoprotein (54 kDa) x x x
	α-2-antiplasmin (55kDa) x x x
	Annexin A1 (39 kDa) x x x x
	Angiotensinogen (53kDa) x x
	Antithrombin-3(53 kDa) x x x
	β-2-glycoprotein 1 (38 kDa) x x
	Bacterial/permeability-increasing protein (49 kDa) x x x x
	Catalase (60 kDa) x x
	Corticosteroide-binding globulin (45 kDa) x x
	Gelsolin (81 kDa) x x
	Glucose-6-phosphate isomerase (63 kDa) x x
	Histidine- rich glycoprotein (60 kDa) x x x
	Hemopexin (52 kDa) x x x x
	Kallistatin (49kDa) x x
	Leucine-rich α-2-glycoproteinLeucine (38 kDa) x
	Lipopolysaccaride-binding protein (53 kDa) x
	Liver carboxylesterase 1 (63 kDa) x x
	Myeloperoxidase (84 kDa) x x
	Plasma protease C1 inhibitor (55 kDa) x x
	Protein disulfide-isomerase (57 kDa) x x x
	Serotransferrin (77 kDa) x x x x
	Serpin B3-B4 (45kDa) x
	Serum Albumin (69 kDa) x x x x
	Serum paraoxonase (40 kDa) x
	Thyroxine-binding globuline (46 kDa) x x
	Transcobalamin-1 (48 kDa) x
	Tubulin α-1B chain (50 kDa) x x
	Other proteins
	Lactoperoxidase (80 kDa) x x
	Basic salivary proline- rich protein 3 (31 kDa) x

3.3.2 Changes in protein profiles during digestion

The protein profiles of the wheat flour and the wheat sourdoughs digested with HGJ at pH 2 and at pH 4, then further with HDJ at pH 7 from normal persons showed that most of the proteins were digested to smaller peptides Mw < 14,4 kDa (Figure 9 A-E). The protein profiles showed no visible differences in proteins Mw >14,4 kDa in the digested wheat flour compared to the digested wheat sourdoughs, and there were no differences to detect between the four wheat sourdoughs.

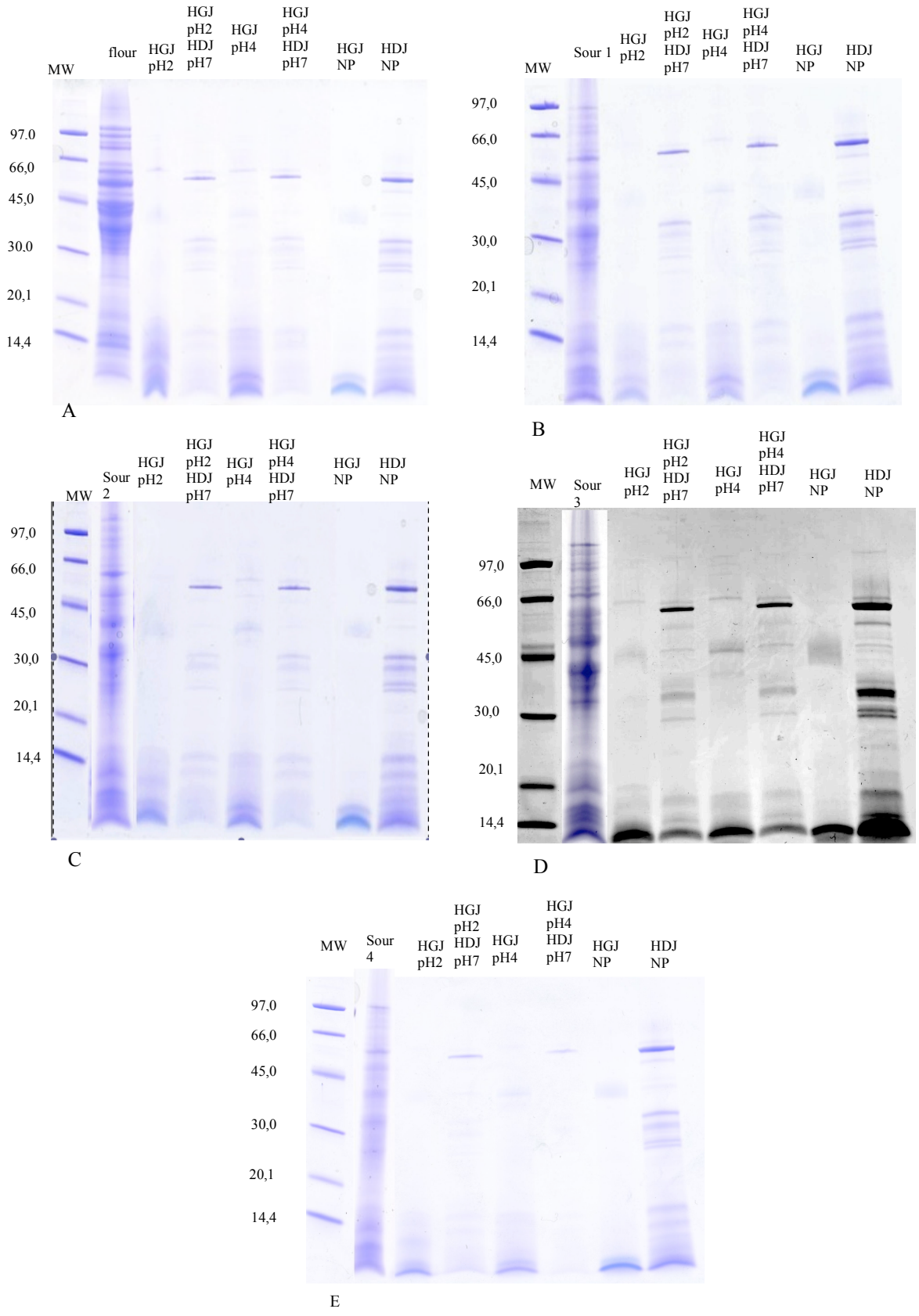


Figure 9: *The SDS-PAGE protein profiles of A) flour, B) Sourdough 1, C) sourdough 2, D) sourdough 3 and E) sourdough 4 digested with gastric juice (GJ) at pH2, GJ at pH2+duodenal juice (DJ) at pH7, GJ at pH4 an GJ at pH4+DJ at pH7 with GJ and DJ from normal persons (NP). The standard (Std) was shown as a molecular weight marker.*

The protein profile of the wheat flour digested with gastric juice (pH 7,4) from celiac patient 2 showed that most of the proteins with Mw between 30,0 kDa and 97,0 kDa remained undigested. The wheat sourdough 3 digested with gastric juice (pH 7,4) from celiac patient 2 showed that some of the proteins were digested in to smaller peptides Mw <14,4 kDa. The wheat flour and the wheat sourdough 3 digested with gastric juice (adjusted to pH2) from celiac patient 2 showed reduced bands. The protein profiles after further digestion of the wheat flour and wheat sourdough 3 with duodenal juice from celiac patient 1 showed that most of the proteins had been digested into smaller peptides <14,4 kDa (Figure 10 A and B).

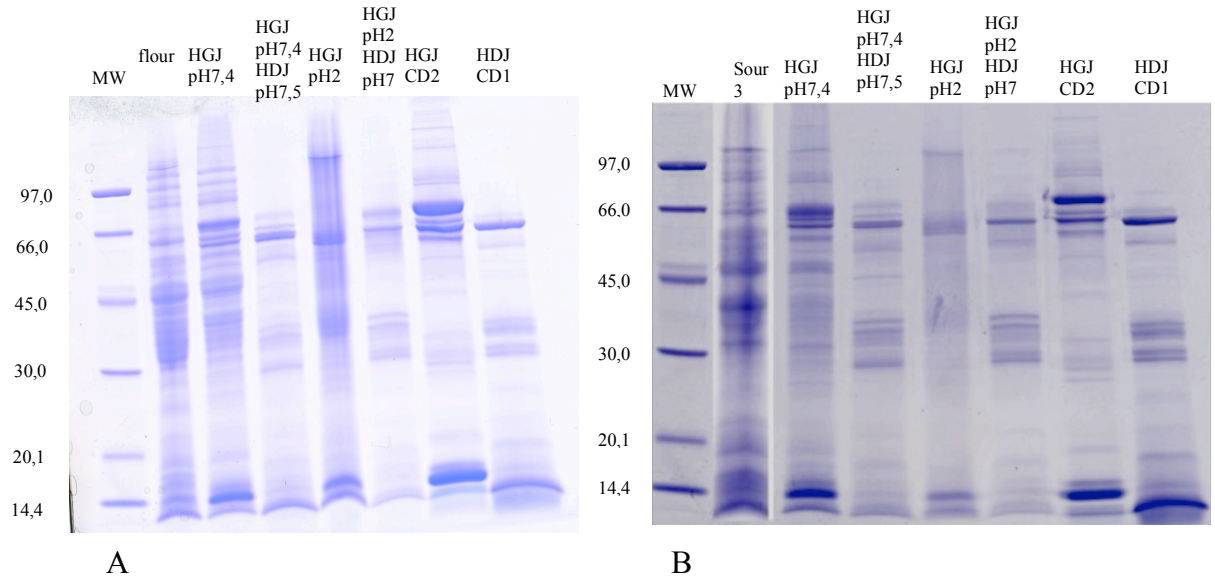


Figure 10: *The SDS-PAGE protein profile of A) wheat flour and B) Wheat sourdough 3, digested with gastric juice (HGJ) with pH7,4, HGJ with pH7,4+ duodenal juice (HDJ) with pH7,5, HGJ at pH2 an HGJ at pH2+HDJ at pH7 with HGJ and HDJ from CD patients. The standard (MW) was shown as a molecular weight marker*

3.3.3 Detection of immune reactive amino acid sequences in gluten proteins and peptides by competitive R5 ELISA

From the standard curve (Figure 11) the amount of immune reactive amino acid sequences in gluten proteins and peptides in wheat flour and wheat sourdoughs were calculated as ng gluten peptides/ml extract. The amount ng/ml was further recalculated into mg/kg. The wheat flour and the four wheat sourdough samples all showed high amount of immune reactive gluten sequences in the UPEX-extracts, 597,7 mg/kg (Table 7). The A_{450nm}, A%, ng/ml immune reactive gluten sequences and mg/kg immune reactive gluten sequences is shown in attachment 1.

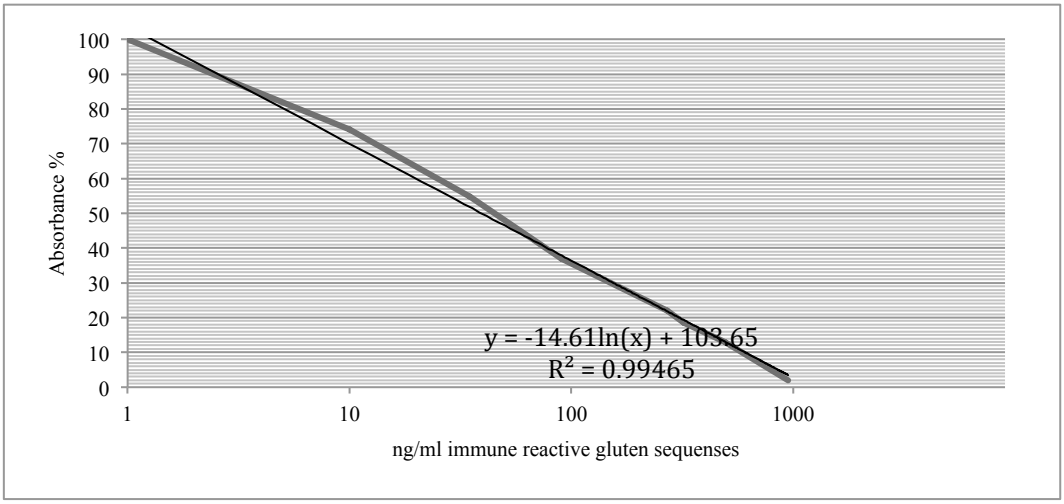


Figure 11: *Standard curve for competitive R5 ELISA.*

Table 7: *Results of the wheat flour and wheat sourdough extracts obtained by the competitive R5 ELISA measured by A_{450nm} and recalculated to mg/kg immune reactive gluten sequences.*

	Extracts				
R5 ELISA	Flour extract	Sourdough 1 extract	Sourdough 2 extract	Sourdough 3 extract	Sourdough 4 extract
Immune reactive gluten sequences (mg/kg)	597,7	597,7	597,7	597,7	597,7

Digestion with human gastrointestinal enzymes from normal persons: The content of immune reactive gluten sequences (mg/kg) in the wheat flour and the wheat sourdoughs digested with gastric juice and duodenal juice from normal persons is shown in Table 8 and Figure 11. The wheat flour digested with gastric juice at pH 2 had a content of 87,4 mg/kg immune reactive gluten sequence, and a content of 183,9 mg/kg after digested with gastric juice at pH 4. After further digestion with duodenal juice at pH 7 the content were 6,9 mg/kg in the sample with gastric juice at pH 2, and 9,8 mg/kg in the sample with gastric juice at pH 4.

The wheat sourdough 1 digested human gastrointestinal enzymes had a content of 37,35 mg/kg immune reactive gluten sequences after digested with gastric juice at pH2, and 51,7 mg/kg after digested with gastric juice at pH 4. After further digestion with duodenal juice pH 7 both samples had a content of 0 mg/kg.

The wheat sourdough 2 digested with gastric juice at pH 2 had a content of 13,2 mg/kg immune reactive gluten sequences, and 46 mg/kg after digested with gastric juice at pH 4. After further digestion with duodenal juice at pH 7 the content were 0 mg/kg in the sample with gastric juice at pH 2, and 2 mg/kg in the sample with gastric juice at pH 4.

The wheat sourdough 3 digested with gastric juice at pH 2 had a content of 15,5 mg/kg immune reactive gluten sequences, and 46 mg/kg after digested with gastric juice at pH 4. After further digestion with duodenal juice at pH 7 the sample with starting point at pH 2 had a content of 2,3 mg/kg, and the sample with starting point at pH 43,4 mg/kg.

The last wheat sourdough digestes with human gastrointestinal enzymes from normal persons was wheat sourdough 4. After digestio with gastric juice at pH 2 had a content of 23 mg/kg immune reactive sequences, while the sample digested with gastirc juice at pH 4 had a content of 89,1 mg/kg. After further digestion with duodenal juice pH at 7 the content were 9,2 mg/kg in the sample with gastric juice at pH 2, and 14,4 mg/kg in the sample with gastric juice at pH 4.

Table 8: *Results of the wheat flour and wheat sourdoughs digested with HGJ and HDJ from normal persons obtained by the competitive R5 ELISA measured by A_{450nm} and recalculated to mg/kg immune reactive gluten sequences.*

R5 ELISA (mg/kg)	HGJ pH 2	HGJ pH 4	HGJ pH2+HDJ pH 7	HGJ pH4+ HDJ pH7
Digested wheat flour	87,4	183,9	6,9	9,8
Digested sourdough 1	37,35	51,7	0	0
Digested sourdough 2	13,2	46	0	2
Digested sourdough 3	15,5	46	2,3	3,4
Digested sourdough 4	23	89,1	9,2	14,4

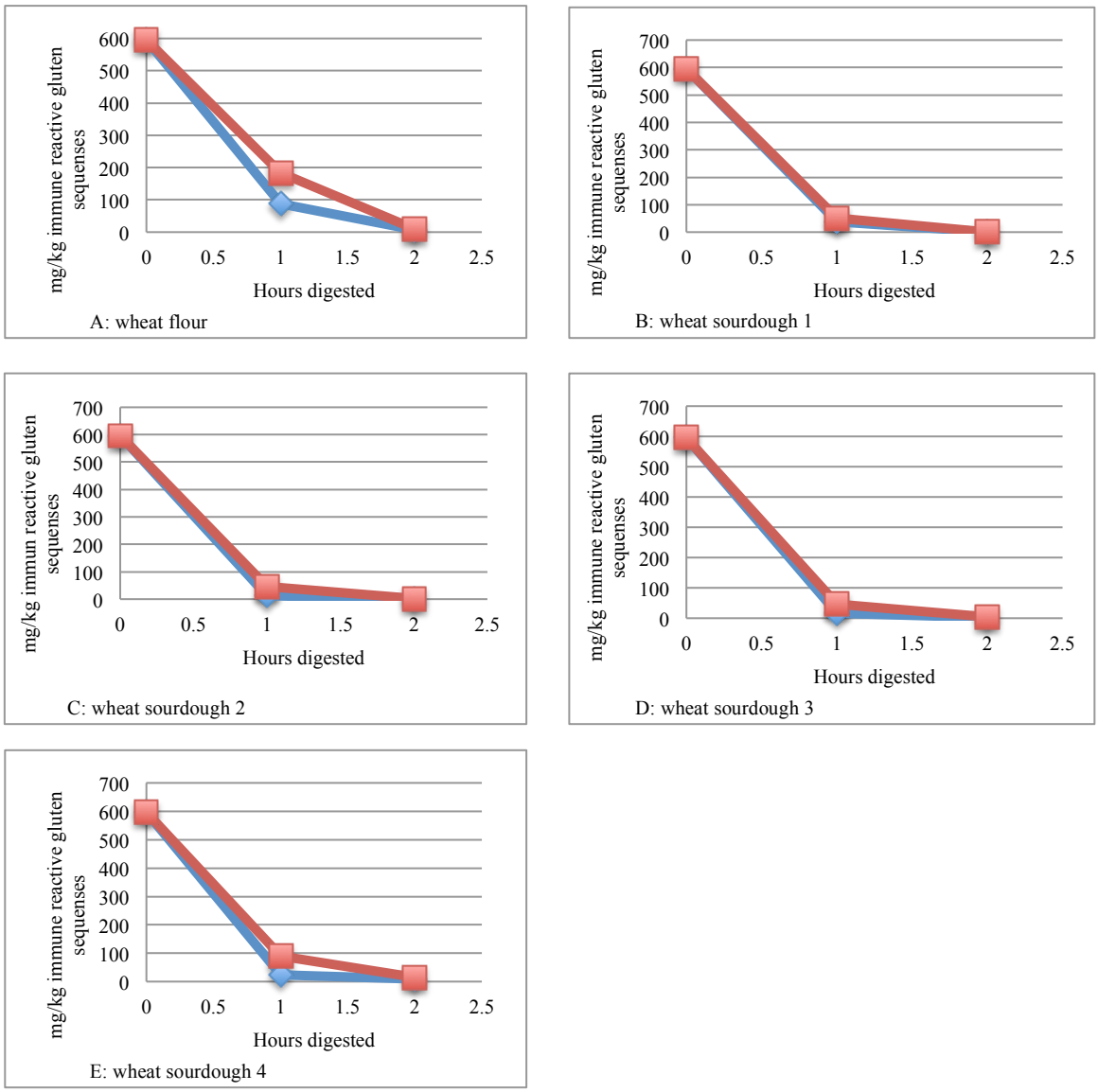


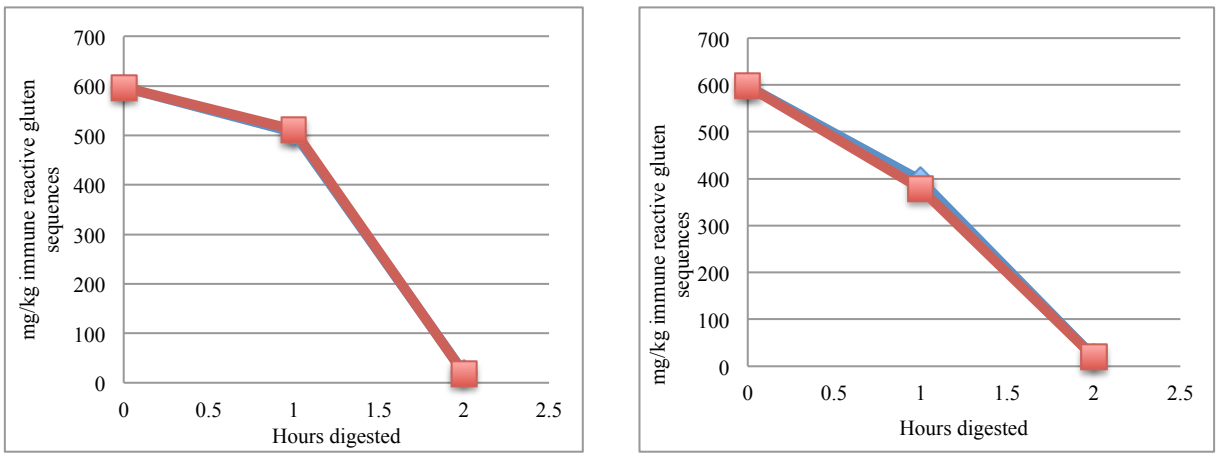
Figure 11: *Content of immune reactive gluten sequences mg/kg after digestion of wheat flour (**A**), Wheat sourdough 1 (**B**), wheat sourdough 2 (**C**), wheat sourdough 3 (**D**) and wheat sourdough 4 (**E**) with HGJ pH 2 (blue line) and pH 4 (red line), and HDJ pH 7 from normal persons.*

The digestion with human gastrointestinal enzymes from celiac patients: The content mg/kg of immune reactive gluten sequences in the wheat flour and wheat sourdough 3 digested with gastric juice from CD2 and duodenal juice from CD1 is shown in Table 9 and Figure 12. The wheat flour digested with gastric juice at pH 7,4 had a content of 505 mg/kg immune reactive gluten sequences, and 511,5 mg/kg after digested with gastric juice at pH 2. After further digestion with duodenal juice at pH 7,5 the content were 20,1 mg/kg, and 18,4 mg/kg in the sample with duodenal juice at pH 7.

The wheat sourdough 3 digested with gastric juice at pH 7,4 had a content of 396,6 mg/kg after immune reactive sequences, and 379,3 mg/kg after digested with gastric juices at pH 2. After further digestion with duodenal juice at pH 7,5 the content were 19,5 mg/kg, and 20,01 mg/kg in the sample with duodenal juice at pH 7.

Table 9: *Results of the wheat flour and sourdough 3 digested with HGJ and HDJ from CD patients obtained by the competitive R5 ELISA measured by A_{450nm} and recalculated as to mg/kg immune reactive gluten sequences.*

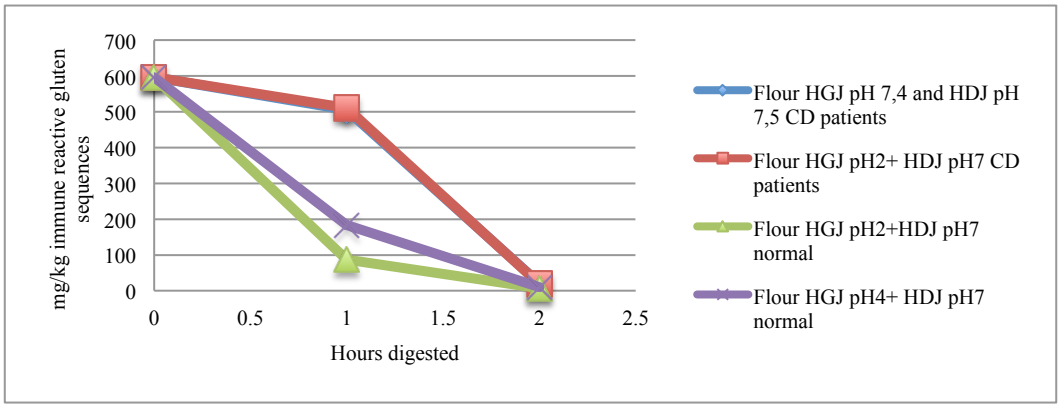
R5 ELISA (mg/kg)	HGJ pH 7,4	HGJ pH2	HGJ 7,4 +HDJ 7,5	HGJ pH 2+ HDJ pH7
Digested wheat flour	505,7	511,5	20,1	18.4
Digested sourdough 3	396,6	379,3	19,5	20,1



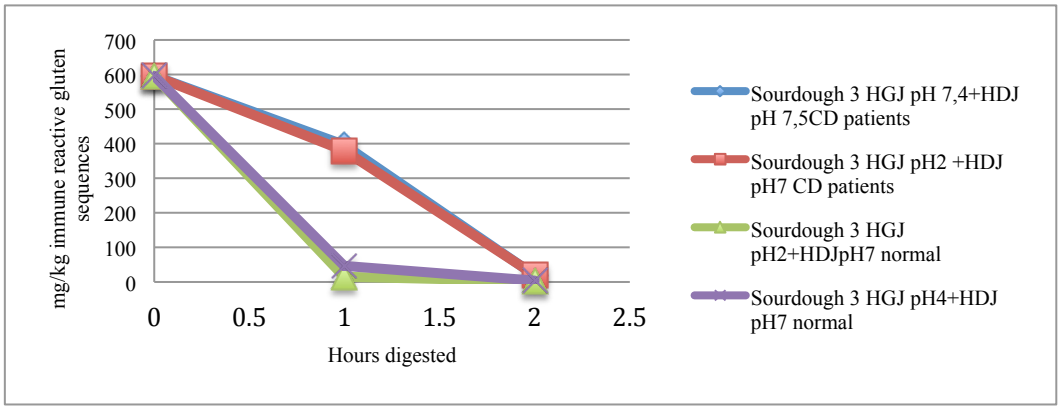
A: wheat flour B: wheat sourdough 3

Figure 12: *Content of immune reactive gluten sequences mg/kg after digestion of wheat flour (**A**) and wheat sourdough 3 (**B**) with HGJ pH 7,4 and HDJ pH 7,5 (blue line) and HGJ pH 2 and HDJ pH 7 (red line) from celiac patients.*

The comparison between normal persons and celiac patients is shown in Figure 13 A and B. The content mg/kg of immune reactive gluten sequences in the wheat flour and the wheat sourdough 3 were much higher after digested with gastric from celiac patient 2, compared to the content mg/kg in the samples digested with gastric juice from normal persons. After further digestion with duodenal juice the content were still higher in the samples digested with duodenal juice from celiac patient 1, than in the sample digested with duodenal juice from normal persons, but the content mg/kg difference were reduced.



A



B

Figure 13: *Content mg/kg of immune reactive gluten sequences after digestion of wheat flour (**A**) and wheat sourdough 3 (**B**) with HGJ and HDJ from normal persons, and HGJ and HDJ from CD patients.*

The comparison of the digestion of wheat flour and wheat sourdoughs is shown in Figure 14. After digested with gastric juice from normal persons and celiac patient 1, the content of immune reactive gluten sequences (mg/kg) was less in wheat sourdoughs samples than in the wheat flour samples. After further digestion with duodenal juice the content of gluten peptides mg/kg in the digested wheat sourdoughs and wheat flour levelled more out, all samples contained <20 mg/kg.

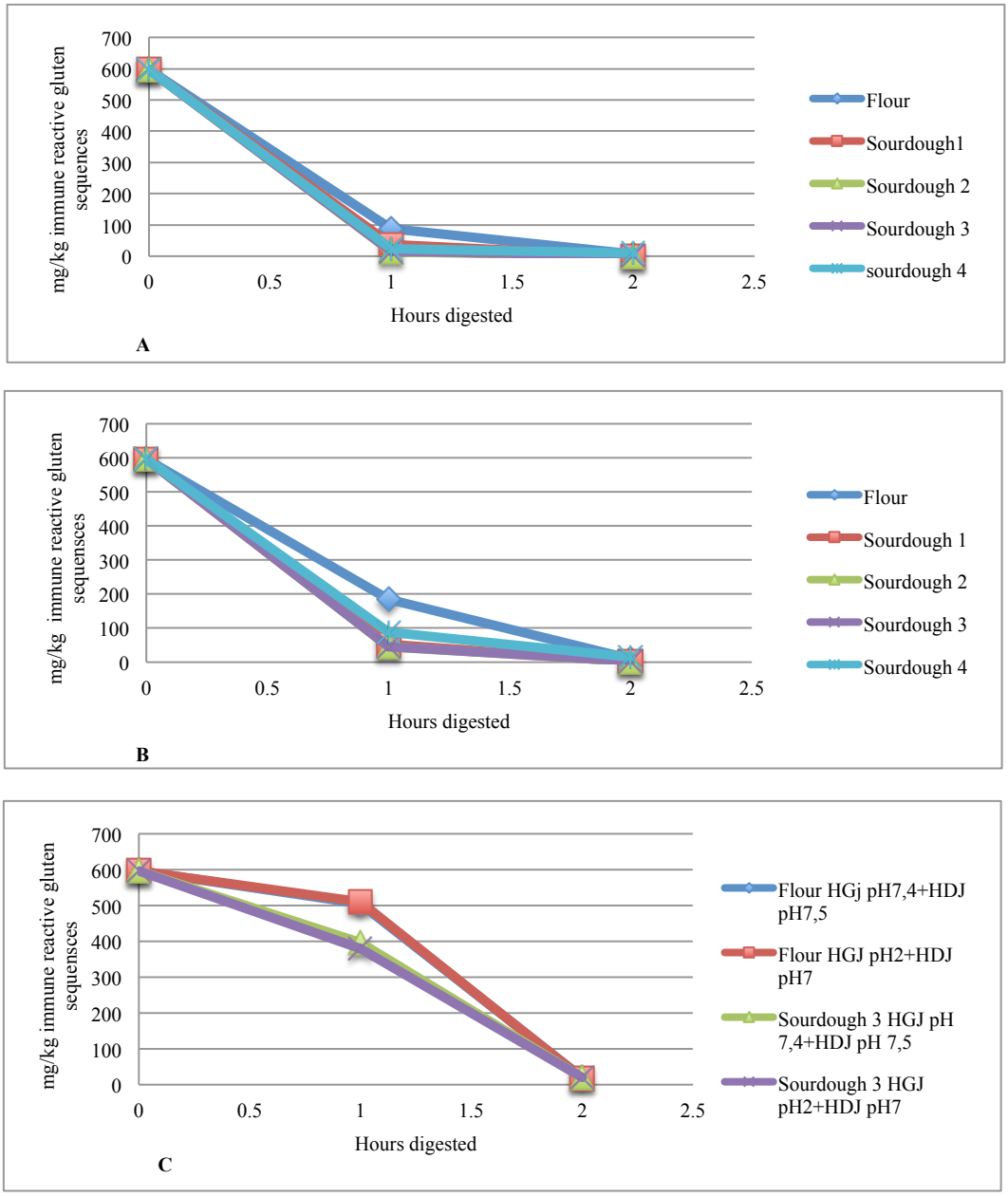


Figure 13: *Content mg/kg of immune reactive gluten sequences after digestion of wheat flour and the wheat sourdoughs with HGJ at pH 2 and HDJ at pH 7 (**A**) and HGJ at pH 4 and HDJ at pH7 (**B**) from normal persons, and digestion of wheat flour and wheat sourdough 3 with HGJ at pH7,4 and HDJ at pH 7,5 and HGJ at pH2 and HDJ at pH 7 from celiac patients (**C**).*

4 Discussion

The present study describes the gluten protein profiles and the content of immune reactive amino acid sequences of gluten protein in wheat flour and four wheat sourdoughs with different starter cultures. It also identifies the protein profile and the content of immune reactive amino acid sequence of gluten protein in the wheat flour and the wheat sourdoughs after *in vitro* digestion with gastric juice (HGJ) and duodenal juice (HDJ) from normal persons, and HGJ from celiac patient 2 (CD2) and HDJ from celiac patient 1 (CD1). The identification of the protein profiles was performed using SDS-PAGE, and the identification of content immune reactive gluten sequence (mg/kg) was performed using the competitive R5 ELISA.

4.1 Protein extraction from wheat flour and wheat sourdough

To be able to analyse the content of immune reactive gluten sequences (mg/kg) in wheat flour and wheat sourdoughs by competitive R5 ELISA the gluten proteins needed to be extracted. In this study two different extraction protocols (ethanol (60%) and UPEX extraction) were tested and compared in terms of yield. The UPEX extraction method has previously been reported to be able to extract 90-100% of total gluten proteins, while the ethanol (60%) extraction method only 9-16%. In both cases the extractions where preformed on gluten-free samples spiked with gluten (Mena. et al., 2012). So as expected, in this study the UPEX extraction method was able to extract more gluten proteins (content of nitrogen (N) measured with the kjeldahl method) than the ethanol (60 %) extraction method. The UPEX extraction method was only able to extract 40-50% of the theoretical calculated amount N in the wheat flour and wheat sourdough. The wheat flour and wheat sourdough used in this study contained gluten in a natural form, with both gliadins and glutenins. The presence of gluten in a natural from means that the gluten network, closely tightened by intra-and interchained disulphide bonds were formed (Wieser, 2007, Uthayakumaran. et al., 2000). The presence of gluten protein in a natural form in the wheat flour and wheat sourdough might be a reason for the low UPEX extraction method not being able to extract 100% of the gluten in this study. The ethanol (60%) extraction method was able to extract 17,3% of the theoretical calculated amount N in the wheat flour, which is in keeping with the results of Mena et al. (2012). From the wheat sourdough it was not possible to extract any N-containing components. The wheat sourdough was viscous which made it hard to dissolve totally in the ethanol (60%) extraction

solution. During dough mixing and preparation the bonds making the gluten network get stronger (Wieser, 2007). The mixing of the flour and water preparing the wheat sourdough may have been the reason for the ethanol (60%) solution not being able to extract any of the proteins. Performing competitive R5 ELISA all the proteins had to be extracted and solubilized. The UPEX extraction method gave better results than the ethanol (60%) extraction method, therefor this method was used to perform the competitive R5 ELISA. The UPEX-extractions of the digested wheat flour and wheat sourdoughs had more clear solution and a smaller pellet than the UPEX-exactions of the undigested wheat flour and wheat sourdoughs. This might have made the gluten proteins and peptides easier to extract after diluted in HGJ and HDJ. But because the total amount of gluten proteins and peptides in the UPEX-extracts of the digested samples were never measured, the amount extracted was uncertain. So, the results based on the competitive R5 ELISA in this study were no more than an indication.

4.2 The gluten proteins profiles of wheat flour and wheat sourdoughs

Lactic acid bacteria (LAB) induced proteolysis of toxic gluten peptides have in recent years been suggested as a method to decrease the toxicity of products made from wheat, barley and rye (Moroni. et al., 2009). Different studies have shown that specific LAB has the ability to hydrolyse gluten proteins during sourdough fermentation (Wieser. et al., 2008, Cagno. et al., 2004). In this study wheat sourdoughs with different LAB were used, *Lactobacillus brevis, Leuconostoc dextranicum* and *Lactobacillius plantarum*. These may have different cell bound proteases that are involved in gluten degradation (Caputo. et al., 2010, Cagno. et al., 2004). The protein profiles of the wheat flour and the wheat sourdoughs showed that there might be one to two protein bands missing in the wheat sourdoughs compared to the wheat flour. These proteins may have been degraded by the starter cultures in the sourdoughs, however, due to many bands and differences in the Coomassie staining it is difficult to document, even after running the gels many times. Since different LAB may have different cell bound proteases, small differences in the protein profile of the wheat sourdoughs were expected. Still, there were no visible differences in the protein profile of the four wheat sourdoughs. The results of the content of immune reactive gluten sequences showed no difference between the wheat flour and the wheat sourdoughs, all had a content of 597,7 mg/kg. Based on these results the wheat sourdoughs it shelf can not be labelled as *low in gluten* or *gluten-free*, since the Norwegian food authority (Mattilsynet) has set the following limit <100 mg/kg and 20 mg/kg.

The two different amount are set based on that celiac patient can tolerate different, small amounts of gluten to limited extent (Helse-ogOmsorgsdepartementet, 2009).

4.3 *In vitro* digestion of wheat flour and wheat sourdoughs

The pH of the environment during digestion affects the activity of proteolyic enzymes. The gastric enzyme pepsin has an optimum pH between 1.6 and 3.2 (Barret. et al., 2010, Untersmayr. and Jensen-Jarolim, 2008). A study reported by Piper and Fenton (1965) showed that at pH 4.5 approximately 70% of maximal peptic activity was still present. Between pH 5 to 6.5-7.5 the pepsin was stable, but had no activity. By adjusting the pH to pH 2, maximal peptic activity could be restored. At pH above pH 7.5 the pepsin was irreversibly inactivated (Piper. and Fenton, 1965). Johnston et al. (2007) confirmed that the pepsin was irreversibly inactivated at pH 8 (Johnston et al., 2007).

In this study the wheat flour and all four wheat sourdoughs were digested with HGJ and HDJ from normal persons. The pH of the HGJ from the batch of 5 normal persons was pH 2,06, which means that according to Barret et al. (2010), and Utersmayr and Jensen-Jarolim (2008) the pepsin should be fully active. The gastric digestion with HGJ from normal persons, were done at pH 2 and pH 4, and further with HDJ at pH 7. The two different pH values of the HGJ were used to see if the pH had an effect on the pepsins ability to digest the gluten proteins. The protein profile after both the gastric digestion and the duodenal digestion of the wheat flour and wheat sourdoughs showed that the relative amount of the different protein were decreasing, meaning that proteins were digested in to smaller peptides Mw <14.4 kDa, not detectible by the SDS-PAGE. This means that the pepsin was active at pH 2 and pH 4. The results of the content of immune reactive gluten sequences (mg/kg) showed that after gastric digestion of wheat flour at pH 2 the content was 87 mg/kg, which was lower than the content 184 mg/kg after digested at pH 4. The same was seen after digestion of all the wheat sourdoughs were the content was between 13 and 38mg/kg after digested at pH 2, and between 46 and 29 mg/kg after digested at pH 4. This is in line with Piper and Fenton (1965) results of the pepsin activity being higher at pH 2, than at pH 4.

In vitro and *In vivo (in rats)* digestion of gluten has previously showed that immune reactive gluten protein and peptides remains largely intact during digestion with pancreatic and brush border membrane enzymes, especially the 33-mer peptide known as “super antigen” (Comino

et al., 2012, Hausch. et al., 2002, Shan. et al., 2002). After further digestion of the wheat flour and wheat sourdoughs with HDJ at pH 7 from normal persons, the content mg/kg of gluten sequences had decreased to < 14 mg/kg. In almost all samples there were still detected a low content (2 to 14 mg/kg). This means that some immune reactive gluten sequences still were intact, indicating that the intestinal enzymes were not able to digest them completely. The monoclonal R5 antibody used in the competitive R5 ELISA interacts with a conseus sequence of five amino acids (glutamine (Q)-X-proline (P)-phenylalanine (F)/tryptophan(W)- proline). Variations of this sequence occurs in celiac-toxic motifs in gluten protein and peptides, causing immune response in celiac patients (Valdés. et al., 2003). Several immune reactive gluten peptides identified, such as α-gliadin: 21-40, α-gliadin: 31-49, α-gliadin: 57-89 (33-mer), γ-gliadin: 60-79 and glutenin: 46-60 (a) contain variations of this conseus sequence. An identification of the immune reactive gluten peptides and proteins detected after digestion in this study was not performed. This means that the identity of the detected gluten peptides and proteins cannot be documented.

Only the wheat flour and wheat sourdough 3 were digested with HGJ and HDJ from CD patients. The wheat flour was chosen because it was showing the gluten proteins at time point zero, before mixed with the wheat sourdoughs containing LAB. The wheat sourdough 3 was chosen based on the starter culture being a mix of the starter cultures in sourdough 1 and sourdough 2, and in that way showed the effect of two different types of LAB. To activate the pepsinogens in the gastric juice, the environment has to have an acidic pH (Richter et al., 1998). In the HGJ used from the CD2 the pH was measured to pH 7.4. According to Piper and Fenton (1965) and Johnston et al. (2007) the pepsin should be inactive at that pH, but just within the limit of getting fully activated by adjusting the HGJ to pH 2. The protein profile after the gastric digestion at pH 7,4 of wheat flour showed that most of the proteins >14,4 kDa were not digested into smaller peptides which was expected with no pepsin activity. Even so, after the gastric digestion with HGJ pH 7.4 from the CD2 the R5 ELISA results showed that a small content of the immune reactive gluten sequences (mg/kg) had been digested (92 mg/kg), which indicated that some proteolysis had happened. The protein profile of wheat sourdough 3 digested with HGJ from CD 2 showed that some proteins were digested into smaller peptides mW <14,4 kDa, and the results of the amount immune reactive gluten sequences digested (201 mg/kg) showed the same. Reflux of HDJ from the duodenum thru pylorus into the ventricle is a normal phenomenon (Heading, 1983, Koek et al., 2005). The liquid

chromatography- mass spectrometry (LC-MS) showed pancreatic enzymes as α-amylase, Carboxypeptidase E precursor, Trypsin and dipeptidyl peptidase in the HGJ from CD2. According to Saladin (2012) the optimum pH for the pancreatic enzymes is pH 6.0- 7.0, which means that the small amount of gluten proteins and peptides digested by the HGJ at pH 7.4 might have been done by pancreatic enzymes refluxed from the duodenum.

The pH in the HGJ from the CD2 was adjusted to pH 2, to see if low pH increased the content immune reactive gluten sequences (mg/kg) digested. The protein profile of the wheat flour and wheat sourdough 3 digested with HDJ from CD1 showed reduced bands, which made the protein profile difficult to analyse. The content immune reactive gluten sequences in samples digested at pH 2 compared to those digested at pH 7.4 were approximately the same for both the wheat flour (512 mg/kg and 506 mg/kg) and wheat sourdough 3 (379 mg/kg and 397 mg/kg). These results were in contrary to Johnston et al. (2007) and Piper and Fenton (1965). According to them the pepsin should have been fully activated, since the pH of the HGJ at starting point was below 7.5. The small amount of immune reactive gluten sequences digested at pH 2 indicates that some of the pepsin might have been reactivated, but compared to the content of immune reactive gluten sequences that were digested with HGJ at pH 2 from normal persons the pepsin cannot have been fully activated.

Despite the low content of immune reactive gluten sequences (mg/kg) digested in the wheat flour and the wheat sourdough 3 during the digestion with HGJ from CD2, the protein profile and the results of the content immune reactive gluten sequences showed that most of the immune reactive gluten sequences were digested to < 20 mg/kg in all the samples with HDJ from CD1. Compared to the content immune reactive gluten sequences detected in the wheat flour and wheat sourdough 3 digested with HGJ pH 7 from normal persons (<14 mg/kg), the difference in content of immune reactive gluten sequences had decreased noticeably. Nevertheless, there were still detected a low content. This indicates that also the intestinal enzymes from CD1 weren't able to completely digest the immune reactive gluten sequences completely.

After digested with HGJ from normal persons and CD2, the wheat sourdough samples had a lower content of the immune reactive gluten sequences 8mg/kg), than the wheat flour samples. This indicates that the LAB may have had some effect on the gluten proteins as studies by Cagno et al. (2004) and Weiser et al. (2008) had shown, making them easier to

digest. After further digested with HDJ the content had decreased and the difference between wheat flour and wheat sourdoughs had levelled.

5 Conclusion

The first aim of this study was to detect differences in the gluten protein profile and the content (mg/kg) of gluten in wheat flour and wheat sourdoughs with different starter cultures. The second was to digest gluten in wheat flour and selected wheat sourdoughs and detect immunogenic proteins and peptides after *in vitro* digestion using human gastrointestinal enzymes from normal persons and from celiac patients

This study showed that the content of immune reactive amino acid sequences of gluten protein and peptides did not differ between the wheat flour and the wheat sourdoughs. But, during the *in vitro* digestion the gluten protein in the wheat sourdoughs were easier to digest than the gluten protein in the wheat flour, for both normal persons and celiac patient 2. Based on these results I conclude that the lactic acid bacteria have had en effect on the gluten proteins in the wheat sourdoughs making them easier to digest, but the effect had not been sufficient enough to label the wheat sourdoughs *low in gluten* or *gluten-free*.

The present study also showed that the content of immune reactive gluten sequences digested during the gastric digestion differed noticeably between normal persons and celiac patient 2. It showed that the gastric juice from normal persons were able to digest a much higher content of gluten protein than the gastric juice from celiac patient 2, probably due to high pH in the gastric juice from celiac patient 2. The results in this study were based on digestion with gastric juice from only one celiac patient. To conclude, gastric juice from more celiac patients needs to be investigated.

6 Future perspectives

In future studies the cell bound enzymes of lactic acid bacteria (LAB) used in sourdough should be investigated and identified. In that way LAB with cell bound enzymes specific for degradation of the peptide bonds in gluten proteins can be used as starter culture in the sourdough. Possibly that can lower the content of immune reactive gluten proteins and peptides in the sourdough.

Based on the results in this study, in the future analysis of gastric juice and duodenal juice from more celiac patients should be investigated to see if the findings in this study was unique for this one celiac patient, or if it´s a common phenomenon among celiac patients.

The extraction used in this study was not extracting 100% of the gluten protein from the wheat flour and the wheat sourdoughs. So in the future an extraction method able to extract up to a 100% of the gluten proteins should be developed.

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ATTACHMENT 1:

Results of the wheat flour and wheat sourdough extracts obtained by the competitive R5 ELISA measured by A_{450nm} and recalculated as A% corresponding to Ng/ml reactive immunogenic peptides

	Extracts				
	Flour extract	Sourdough 1 extract	Sourdough 2 extract	Sourdough 3 extract	Sourdough 4 extract
A_{450nm}	0,0446	0,0445	0,0441	0,0463	0,0436
A (%)	1,9	1,9	1,9	1,9	1,9
Immune reactive gluten sequences (ng/ml)	525000	525000	525000	525000	525000
Immune reactive gluten sequences (mg/kg)	597,7	597,7	597,7	597,7	597,7

Results of the wheat flour digested with HGJ and HDJ from normal persons obtained by the competitive R5 ELISA measured by A_{450nm} and recalculated as A% corresponding to Ng/ml reactive immunogenic peptides

	Digested wheat flour			
Normal persons	HGJ pH 2	HGJ pH 4	HGJ pH 2+HDJ pH7	HGJ pH4+ HDJ pH7
A_{450nm}	0,72	0,432	1,659	1,561
A (%)	31	18,5	70,9	66,7
Immune reactive gluten sequences (ng/ml)	76000	160000	6000	8500
Immune reactive gluten sequences (mg/kg)	87,4	183,9	6,9	9,8

Results of the wheat sourdough 1 digested with HGJ and HDJ from normal persons obtained by the competitive R5 ELISA measured by A_{450nm} and recalculated as A% corresponding to Ng/ml reactive immunogenic peptides

	Digested sourdough 1			
Normal persons	HGJ pH 2	HGJ pH 4	HGJ pH 2 + HDJ pH 7	HGJ pH 4 + HDJ pH 7
A_{450nm}	1,0	0,849	2,511	2,51
A (%)	42,7	36,28	107,3	107,2
Immune reactive gluten sequences (ng/ml)	32500	45000	0	0
Immune reactive gluten sequences (mg/kg)	37,35	51,7	0	0

Results of the wheat sourdough 2 digested with HGJ and HDJ from normal persons obtained by the competitive R5 ELISA measured by A_{450nm} and recalculated as A% corresponding to Ng/ml reactive immunogenic peptides

	Digested sourdough 2			
Normal persons	HGJ pH 2	HGJ pH 4	HGJ pH 2 + HDJ pH 7	HGJ pH 4 + HDJ pH 7
A_{450nm}	1,438	0,927	2,398	2,23
A (%)	61,5	39,6	102,5	95,3
Immune reactive gluten sequences (ng/ml)	11500	40050	0	1,1
Immune reactive gluten sequences (mg/kg)	13,2	46	0	2

Results of the wheat sourdough 3 digested with HGJ and HDJ from normal persons obtained by the competitive R5 ELISA measured by A_{450nm} and recalculated as A% corresponding to Ng/ml reactive immunogenic peptides

	Digested sourdough 3			
Normal persons	HGJ pH 2	HGJ pH 4	HGJ pH 2 + HDJ pH 7	HGJ pH 4 + HDJ pH 7
A_{450nm}	1,334	0,913	2,053	1,956
A (%)	57	39	87,7	83,6
Immune reactive gluten sequences (ng/ml)	13500	40000	2000	3000
Immune reactive gluten sequences (mg/kg)	15,5	46	2,3	3,4

Results of the wheat sourdough 4 digested with HGJ and HDJ from normal persons obtained by the competitive R5 ELISA measured by A_{450nm} and recalculated as A% corresponding to Ng/ml reactive immunogenic peptides

	Digested sourdough 4			
Frisk	HGJ pH 2	HGJ pH 4	HGJ pH 2 + HDJ pH 7	HGJ pH 4 + HDJ pH 7
A_{450nm}	1,204	0,691	1,54	1,47
A (%)	51,5	29,50	65,80	63
Immune reactive gluten sequences (ng/ml)	20000	77500	8000	12500
Immune reactive gluten sequences (mg/kg)	23	89,1	9,2	14,4

Results of the wheat flour digested with HGJ and HDJ from celiac patients obtained by the competitive R5 ELISA measured by A_{450nm} and recalculated as A% corresponding to Ng/ml reactive immunogenic peptides

	Digested wheat flour			
CD patients	HGJ pH 7,4	HGJ pH2	HGJ 7,4 +HDJ 7,5	HGJ pH 2+ HDJ pH7
A_{450nm}	0,054	0,07	1,351	1,392
A (%)	3,20	3	58	59
Immune reactive gluten sequences (ng/ml)	440000	445000	17500	16000
Immune reactive gluten sequences (mg/kg)	505,7	511,5	20,1	18.4

Results of the wheat sourdough 3 digested with HGJ and HDJ from celiac patients obtained by the competitive R5 ELISA measured by A_{450nm} and recalculated as A% corresponding to Ng/ml reactive immunogenic peptides

	Digested sourdough 3			
CD patients	HGJ pH 7,4	HGJ pH 2	HGJ 7,4+HDJ 7,5	HGJ pH2+HDJ pH7
A_{450nm}	0,193	0,196	1,37	1,342
A (%)	8,2	8,4	58,5	57,4
Immune reactive gluten sequences (ng/ml)	345000	330000	17000	17500
Immune reactive gluten sequences (mg/kg)	396,6	379,3	19,5	20,1

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