SATUMARJA STENMAN

Coeliac Disease-inducing Gluten

In vitro harmfulness and detoxification by germinating cereal enzymes

■

ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the Auditorium of Finn-Medi 5,
Biokatu 12, Tampere, on February 25th, 2011, at 12 o'clock.

UNIVERSITY OF TAMPERE

UNIVERSITY
OF TAMPERE

ACADEMIC DISSERTATION
University of Tampere, Medical School
Tampere University Hospital, Department of Paediatrics and Department of Gastroenterology and Alimentary Track Surgery
Finland

Supervised by
Docent Katri Kaukinen
University of Tampere
Finland
Docent Katri Lindfors
University of Tampere
Finland

Reviewed by
Professor Frits Koning
Leiden University
The Netherlands
Docent Aki Manninen
University of Oulu
Finland

Distribution
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 40 190 9800
Fax +358 3 3551 7685
taju@uta.fi
www.uta.fi/taju
http://granum.uta.fi

Cover design by
Mikko Reinikka

Acta Universitatis Tamperensis 1581
ISBN 978-951-44-8318-9 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 1034
ISBN 978-951-44-8319-6 (pdf)
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2011

ABSTRACT

Coeliac disease is an autoimmune disorder triggered by prolamins of wheat, rye and barley (gliadin, secalin and hordein, respectively). The high content of proline and glutamine residues in the gluten prolamins renders these prolamins highly resistant to human digestive enzymes. In genetically susceptibile individuals this is thought to lead to activation of innate and adaptive immune responses in the small-intestinal mucosa and development of coeliac disease, characterised by mucosal villous atrophy and crypt hyperplasia together with the presence of disease-specific IgA-class autoantibodies. The lifetime disease can currently be treated only by avoidance of wheat, rye and barley, a gluten-free diet.

The purpose of the present study was first to demonstrate the gluten-dependent activation of innate and adaptive immune reactions in several *in vitro* models in order to investigate the harmfulness of wheat gliadin and rye secalin related to coeliac disease *in vitro* (**I**, **II**, **III**). It was demonstrated that gliadin and secalin equally stimulate innate immunity-related reactions in Caco-2 epithelial cells (**II**, **III**), suggesting that rye secalin is as toxic as wheat gliadin also in thr early phases of the disease mechanisms and thus preferably to be exluded from the diet of patients suffering from coeliac disease. Moreover, gliadin was observed to stimulate proliferation of coeliac patient-derived small-intestinal mucosal T cells (**II**) and to activate disease-specific adaptive immune reactions in small-bowel mucosal biopsies from coeliac disease patients (**I**, **II**). This series further evaluated the relevance of the human small-intestinal organ culture method in the field of coeliac disease research in general (**I**). It was observed that biopsy samples from untreated or short-term-treated coeliac disease patients still retaining small-bowel mucosal IgA deposits should be used in order to reliably study the toxic effects of gluten *ex vivo* (**I**).

Furhermore, with a view to develop alternative treatments for coeliac disease by fully detoxifying gluten peptides already prior to their entrance into the small-bowel mucosa, gliadin and secalin were cleaved with germinating cereal enzymes derived from wheat, rye and barley (**II**, **III)**. These proteases naturally effect total hydrolysis of gluten prolamins during the germination process in cereal seeds. In the present studies, gliadin and secalin were efficiently degraded into short fragments by these enzymes. In addition, a reduction in the toxicity of enzymatically pre-treated gliadin and secalin products was observed using the above mentioned coeliac disease-related *in vitro* models (**II**, **III)**.

In the current studies, gluten toxicity related to coeliac disease *in vitro,* was evaluated using several overlapping models, including epithelial cells representing innate immunity, T cells related to adaptive immunity, as well as these two linked together in a human organ culture system. These models, gave indications that in the future it will be possible to develop novel medical treatments for the condition by degrading gluten peptides into non-toxic fragments by means of a variety of germinating cereal enzymes. These enzymes may also be relevant in improving the quality and taste of coeliac-safe food products, for example by adding enzymatically pre-treated rye to gluten-free products.

TIIVISTELMÄ

Keliakia on autoimmuunivälitteinen sairaus, jonka aiheuttaa vehnän, rukiin ja ohran prolamiinit (gliadiini, sekaliini, hordeiini). Kyseisten prolamiinien korkea proliini- ja glutamiinipitoisuus tekee näistä hyvin vaikeita hajotettavia ihmisen ruoansulatuskanavan entsyymeille. Tietyn genetiikan omaavilla yksilöillä, tämän ajatellaan käynnistävän ohutsuolen limakalvolla synnynnäisen ja hankinnaisen immunipuolustusreaktion aktivoitumiseen ja keliakian kehittymiseen, mitä kuvaa limakalvon villusten atrofia ja kryptahyperplasia sekä sairaudelle spesifisten IgA-luokan vasta-aineiden ilmestyminen. Elinikäinen sairaus voidaan tällä hetkellä hoitaa vain välttämällä vehnää, ruista ja ohraa eli gluteenittomalla ruokavaliolla.

Tutkimuksen ensimmäinen tavoite oli näyttää gluteenista riippuvien luonnollisen ja hankitun immuniteetin aktivoituminen useissa *in vitro*-malleissa jotta vehnän gliadiinin ja rukiin sekaliinin haittavaikutuksia voitaisiin luotettavasti tutkia *in vitro* (**I**, **II**, **III**). Tutkimuksessa osoitettiin että gliadiini ja sekaliini aiheuttavat samanlaisia synnynnäisiä immuniteettireaktioita Caco-2 epiteelisoluissa (**II**, **III**), mikä puhuu sen puolesta että rukiin sekaliini on yhtä haitallista kuin vehnän gliadiini myös taudin syntymekanismien varhaisissa vaiheissa, eli sekaliini on suositeltavaa poistaa keliakiapotilaiden ruokavaliosta. Lisäksi gliadiini kiihdytti keliakiapotilailta peräisin olevien ohutsuolen limakalvon T-solujen jakautumista (**II**) ja aktivoi keliakiaspesifisen hankitun immuniteetin keliakiapotilaiden ohutsuolen limakalvon koepaloissa (**I**, **II**). Tämä tutkimus myös arvioi ihmisen ohutsuolen kudosviljelymenetelmän toimivuutta yleisesti keliakiatutkimuksen yhteydessä (**I**). Huomattiin, että tutkimuksissa tulisi käyttää hoitamattomien tai lyhyen aikaa hoidettujen keliakiapotilaiden koepaloja, joilla on yhä IgA-kertymiä ohutsuolen limakalvolla, jotta gluteenin haitallisia vaikutuksia voidaan tutkia luotettavasti *ex vivo* (**I**).

Jotta keliakiaan voitaisiin kehittää vaihtoehtoisia hoitomuotoja jossa gluteenipeptidit ovat kokonaan tehty haitattomiksi jo ennen kuin ne kulkeutuvat ohutsuolen limakalvolle, gliadiini ja sekaliini pilkottiin idätettyjen vehnän, rukiin ja ohran entsyymeillä (**II**, **III)**. Luonnossa nämä proteaasit hajottavat gluteenin prolamiineja viljan jyvien itämisen aikana. Tutkimuksissa gliadiini ja sekaliini pilkkoutuivat kyseisten entsyymien avulla tehokkaasti lyhyiksi peptideiksi. Lisäksi entsyymikäsittely vähensi gliadiinin ja sekaliinin haitallisuutta yllä mainituissa keliakiaa kuvailevissa *in vitro* malleissa (**II**, **III)**.

Kyseisissä tutkimuksissa gluteenin haitallisuutta keliakiassa *in vitro* selvitettiin erilaisilla malleilla: epiteelisoluilla jotka kuvaavat luonnollista immuniteettia, hankittua immuniteettia edustavilla T-soluilla sekä näiden yhdistelmällä, ihmisen kudosviljelymenetelmällä. Näiden mallien avulla ehdotettiin, että tulevaisuudessa on mahdollista kehittää sairauteen uusi lääkepohjainen hoito hajottamalla gluteenipeptidit vaarattomaan muotoon käyttämällä yhdistelmää idätettyjen viljojen entsyymejä. Nämä entsyymit voisivat olla myös hyödyllisiä kehitettäessä keliaakikoille tarkoitettujen elintarvikkeiden laatua ja makua kuten lisäämällä entsyymikäsiteltyä ruista gluteenittomiin tuotteisiin.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications **I-III**:

I Stenman SM, Lindfors K, Korponay-Szabo IR, Lohi O, Saavalainen P, Partanen J, Haimila K, Wieser H, Mäki M and Kaukinen K. Secretion of celiac disease autoantibodies after in vitro gliadin challenge is dependent on small-bowel mucosal transglutaminase 2-specific IgA deposits. BMC Immunology 2008;9:6.

II Stenman SM, Venäläinen JI, Lindfors K, Auriola S, Mauriala T, Kaukovirta-Norja A, Jantunen A, Laurila K, Qiao SW, Sollid LM, Männistö PT, Kaukinen K and Mäki M. Enzymatic detoxification of gluten by germinating wheat proteases: Implications for new treatment of celiac disease. Ann Med 2009;41(5):390-400.

III Stenman SM, Lindfors K, Venäläinen JI, Hautala A, Männistö PT, Garcia-Horsman JA, Kaukovirta-Norja A, Auriola S, Mauriala T, Mäki M and Kaukinen K. Degradation of celiac disease-inducing rye secalin by germinating cereal enzymes - diminishing toxic effects in intestinal epithelial cells. Clin Exp Immunol. 2010;161(2):242-9.

ABBREVIATIONS

AN-PEP	prolyl endoprotease from *Aspergillus Niger*
APC	antigen presenting cell
BSA	bovine serum albumin
Caco-2	human colorectal adenocarcinoma cell line
CD	coeliac disease
CD71	transferrin receptor
CXCR3	G protein-coupled receptor 3 in the chemokine receptor family
DPPIV	dipeptidyl peptidase IV from *Aspergillus oryzae*
EB-B2	endoprotease 2 from barley
EC_{50}	half-maximal effective concentration
ELISA	enzyme-linked immunosorbent assay
EmA	endomysial antibody
EP-B2	barley endoprotease B
FITC	fluorescein isothiocyanate-conjugation
FOXP3	forkhead box P3 gene
GFD	gluten-free diet
HLA	human leukocyte antigen
HMW	high molecular weight
HPLC-MS	high-performance liquid chromatography and mass spectroscopy
IFN-γ	interferon-gamma
IgA	immunoglobin A
IEL	intraepithelial lymphocyte
IL	interleukin
JAM	junctional adhesion molecules
LMW	low molecular weight
MHC	major histocompatibility complex

MICA	MHC class 1 chain-related gene A
MICB	MHC class 1 chain-related gene B
MS/MS	tandem mass spectrometry
MYO9B	myosin 9B molecule
NKG2D	natural killer cell group 2 member D receptor
PEP	prolyl endopeptidase
PT	pepsin and trypsin treatment
PT-BSA	pepsin and trypsin-digested bovine serum albumin
PT-G	pepsin and trypsin-digested wheat gliadin
PT-S	pepsin and trypsin-digested rye secalin
SDS-PAGE	sodium-dodecyl sulphate polyacrylamide gel electrophoresis
T84	human colon carcinoma cell line
TCC	T cell clone
TCL	T cell line
TER	transepithelial resistance
TG2	transglutaminase 2
TG2-ab	transglutaminase 2 antibodies
TGF-β	transforming growth factor-beta
TRITC	rhodamine-conjugation
TNF-α	tumor necrocis factor-alpha
ZO-1	zonula occludens-1 protein in epithelial tight junctions

INTRODUCTION

The unique composition of cereal prolamins in wheat, barley and rye renders them resistant to human gastrointestinal proteolytic enzymes. This is due mainly to their unusually high content of glutamine and proline residues, which leads to incomplete degradation of these proteins during normal human digestion (Shan et al., 2002). Such partial degradation is nowadays thought to be the key element in the activation of the immune response in the small-bowel mucosa and the progression of coeliac disease in genetically susceptible persons.

Coeliac disease is an autoimmune-mediated disorder of the small-intestine characterised by gluten-dependent, gradually developing villous atrophy and crypt hyperplasia together with local inflammation in the small-bowel mucosa. In addition, the presence of circulating and small-bowel mucosal autoantibodies is markedly related to the disease. These immunoglobin A (IgA)-class antibodies are produced in the small-bowel mucosa (Picarelli et al., 1996) and targeted against an endogenous enzyme, transglutaminase 2 (TG2) (Dieterich et al., 1997). The lifetime disease can be treated only by strict exclusion of the cereal prolamins (gliadin, hordein and secalin), termed gluten, in the context of coeliac disease. In practice, however a number of formidable problems are encountered with a restricted diet, and a search for alternative treatment strategies is clearly warranted.

One restricting factor in the field of coeliac disease research is the continued lack of an operative animal model for the disease, which would fully correlate with the disease phenotype in humans. Most studies rely on diverse *in vitro* systems including several intestinal epithelial cell lines, coeliac patient-derived disease-specific T cell lines (TCL) and clones (TCC), as well as small-bowel mucosal biopsies maintained in tissue culture. Regardless of the fact that these methods are widely used among researchers, there has been controversy as to whether the small-intestinal mucosal biopsy culture is a relevant tool to study gluten-induced reactions

in coeliac disease *in vitro*. The present series aimed to demonstrate gluten toxicity in different *in vitro* models and to evaluate the relevance of the human small-intestinal organ culture method as a model for gluten toxicity in coealic disease.

It is generally assumed that the pathogenesis of coeliac disease is divided into two distinct pathways, namely innate and adaptive immunity. Of these, the innate pathway is directly stimulated by several gluten fragments termed toxic gluten peptides. In contrast, activation of adaptive immunity is dominated rather by deamidated immunogenic gluten peptides. It has been shown that the toxic and immunogenic peptides are found not only in wheat but also in rye, barley and to some extent also in oats (Vader et al., 2002b; Vader et al., 2003). However, only a few studies have demonstrated the magnitude of toxicity in rye and barley prolamins *in vivo* and *in vitro* in coeliac disease. One further aim of the current study was thus to investigate the *in vitro* toxicity of rye secalin in human intestinal epithelial cells in comparison to wheat gliadin.

Since gluten peptides are incompletely cleaved in the human digestive system, enzyme supplements have been proposed as a novel approach, the object being to accelerate the complete breakdown of gluten epitopes in advance of their absorption in the small-bowel mucosa (Shan et al., 2002). We now introduced a natural means for gluten degradation by pre-treating wheat gliadin and rye secalin with a whole mixture of germinating cereal enzymes from wheat, oats or barley. These enzymes are meant for total cleavage of storage proteins in cereal kernels (Shewry et al., 1995). In the future this method might be utilised as a novel medical treatment for coeliac disease or in food processing in order to develop high-quality coeliac-safe products.

REVIEW OF THE LITERATURE

1. Cereal grains

Since the agricultural revolution about 10 000 years ago, cereals have been one of the mankind's most important global food sources. The most relevant cereal species are wheat, rice and maize, whereas others are consumed mainly regionally. Of these, the grass tribe *Triticeae* includes wheat, rye and barley, which bear particularly close relations to each other (Kasarda, 1997; Shewry et al., 1999). Interestingly, one of the cereal crops, oats, is taxonomically further removed from the *Triticeae* cereals (Figure 1) albeit having very similar appearance and environment for growth.

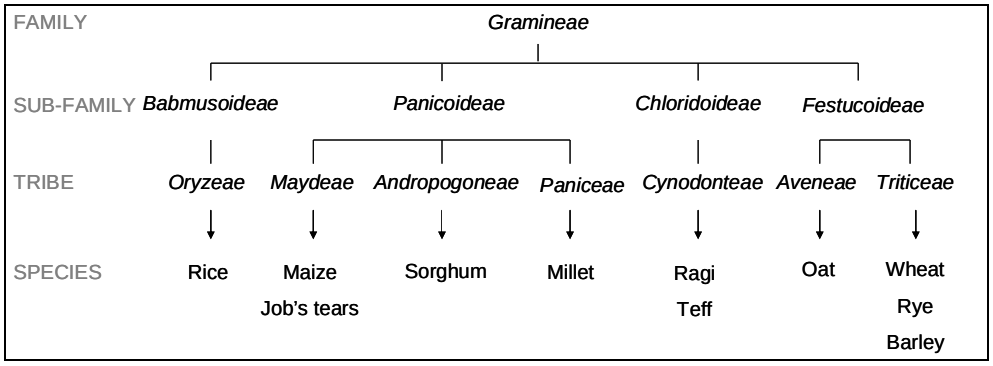

Figure 1. Taxonomic relationships among the most important cereals. This figure is based on original publications (Kasarda, 1997; Shewry et al., 1999).

1.1 The prolamins of the *Triticeae* cereals

Cereal proteins can be classified according to their solubility into different solvents (Osborne, 1907). The four protein groups in wheat are the albumins soluble in water, the globulins soluble in salt solutions, alcohol-soluble prolamins known as the gliadins, and the glutenins soluble in dilute acids. The glutenin proteins can be further divided into subunits of high molecular weight (HMW) and low molecular weight (LMW). These subunits form polymers, linked by disulphide bonds and responsible for the baking quality of wheat flour in forming cohesive, elastic dough when mixed with water (Shewry et al., 2001). Gliadins are monomeric and can be separated into α/β-, γ- and ϖ-gliadins based on their amino acid composition (Kasarda et al., 1976; Wieser, 1994). Rye and barley contain similar kinds of prolamins called secalins and hordeins (Figure 2). About half of the total proteins in wheat, rye and barley are prolamins, in contrast to other cereals such as oats, where the overall prolamin content is markedly lower (5-10%) (Shewry et al., 1995). In oats, the major storage proteins are the salt-soluable globulins, whereas alcohol-soluble avenin represents only a minor element.

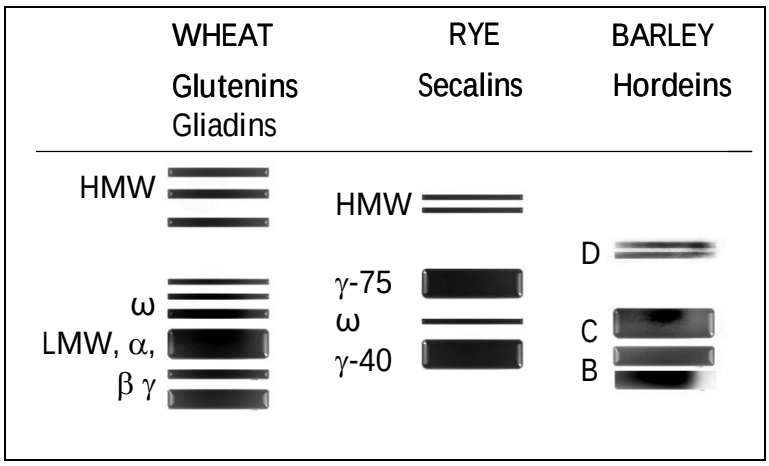

Figure 2. The prolamins in wheat, rye and barley. Modified from original data (Loponen unpublished).

1.2 Germination of grains

Gliadin, secalin and hordein are storage proteins which provide grains with nitrogenous nutrients serving as a major support for the growth of the young seedling in a process termed germination. In the resting cereal seed, the storage proteins are located mostly in the endosperm. During germination these endosperm storage proteins are transported through the scutellum to the embryo, being in the process cleaved into short fragments and finally into single amino acids by a variety of proteolytic enzymes in the grain itself. These enzymes are evolutionarily selected for total degradation of the storage proteins during the germination of kernels (Figure 3) (Galili et al., 1993; Shewry et al., 1995).

Barley proteases have been studied most intensively due to their abundant use in the malting and brewing industry, but in general all cereals share similarities in enzyme content. The four main groups of germinating cereal proteases are cysteine proteinases, aspartic proteinases, serine proteinases and metallo proteinases (Jones, 2005). The catalytic mechanisms of these classes differ; however, it has been estimated that the cysteine proteinases have the most important role in the overall hydrolysis of wheat and barley storage proteins (Bottari et al., 1996), whereas aspartic proteases are equally responsible for the breakdown of rye storage proteins (Brijs et al., 2002; Tuukkanen et al., 2005). In addition, an arsenal of other endoproteases such as proline-specific serine carboxypeptidases play a significant role in the degradation taking place in nature. Each protease has a specific optimal pH, being thus active in a certain phase of the germination process. The majority of them actually operate at low pH. Furhermore, cereal seeds are known to be colonised by resident bacteria, having their own characteristic set of proteases, also possibly play a role in the degradation process (Laitila et al., 2007). However, the exact number of proteolytic enzymes involved and the nature of the process of biochemical degradation of storage proteins remain to be established.

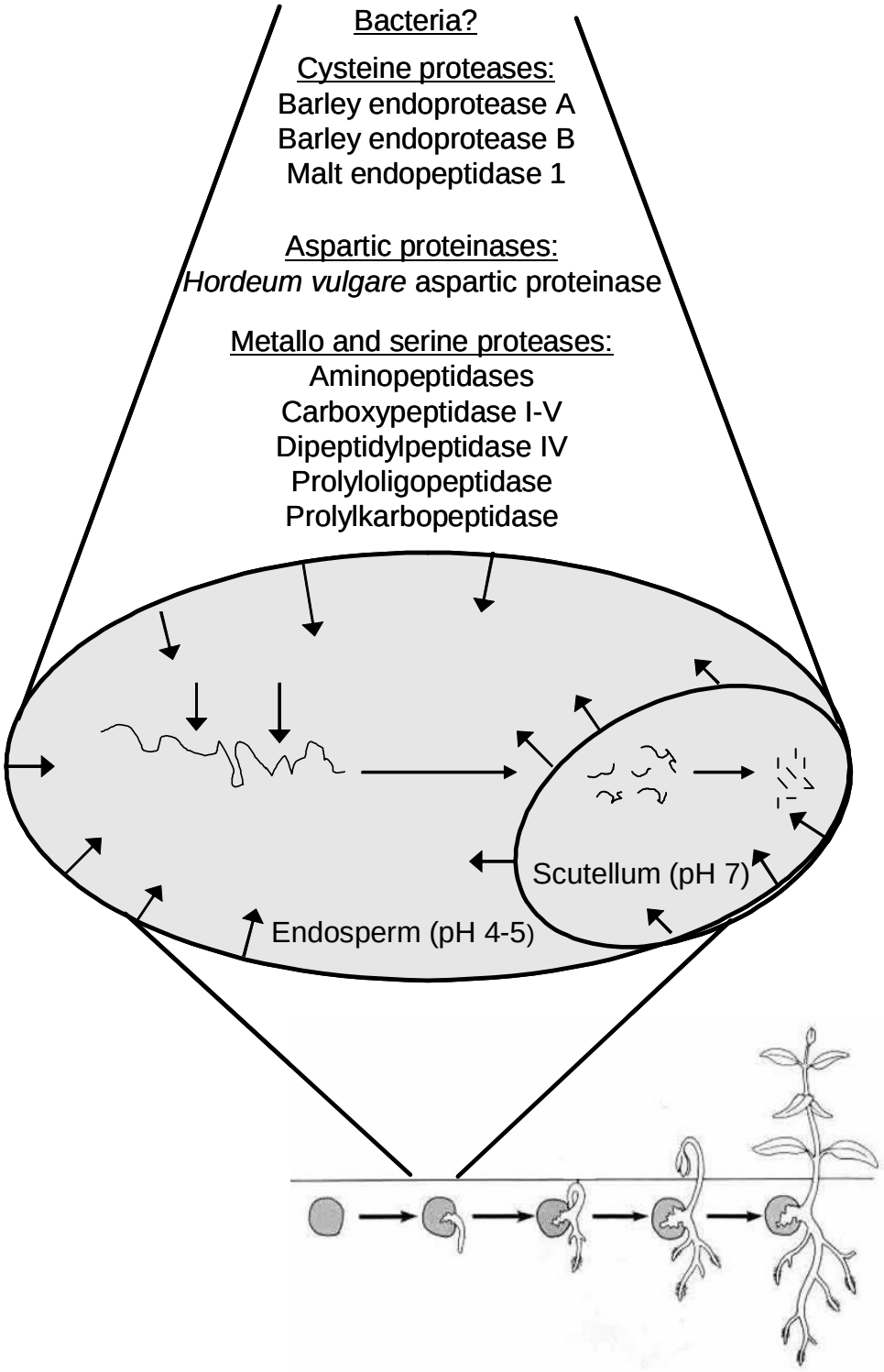

Figure 3. Degradation of cereal storage proteins during germination of kernels. A list of the most important proteolytic enzymes involved in the hydrolysis process. The figure is based on original publications (Mikola, 1986; Jones, 2005).

2. Coeliac disease

The cereals have long represented an important worldwide source of nutritients. In the 20th century, however, Dicke discovered that wheat and rye also have deleterious effects in some individuals, being linked to the disorder known as coeliac disease (Dicke, 1950). A few years later the toxic component involved was identified as a protein fraction of grains, called gluten (Van De Kamer et al., 1953) whereafter the notion of a gluten-free diet (GFD) originated as treatment for the wheat-induced disease. Due to the protein homology of wheat, rye, barley and oats, all were excluded from the coeliac diet. Subsequently the dietary boundaries were revised, oats being allowed when confirmed safe for coeliac disease patients in several studies (Janatuinen et al., 1995; Hogberg et al., 2004; Holm et al., 2006).

Although other cereals have also been found to contain several potentially harmful sequences (Marsh, 1992; Rocher et al., 1996; Vader et al., 2003), wheat and oats have dominated the investigations. Other cereals have been studied mainly in a few early case reports where coeliac patients developed symptoms and small-bowel mucosal histological alterations after ingestion of rye and barley (Dicke et al., 1953; Rubin et al., 1962; Baker and Read, 1976; Anand et al., 1978). More recently, with means available for culturing patient-derived small-bowel mucosal biopsy samples and T cell lines, both rye secalin and barley hordein have been shown to activate T cell-mediated adaptive immune reactions in the small-bowel mucosa *in vitro* (Bracken et al., 2006; Kilmartin et al., 2006). In spite of the sparse evidence, the harmful effects of rye and barley in coeliac disease are in practice assumed on the basis of their close relations to wheat.

2.1 Clinical characteristics

Coeliac disease was long regarded as a disease of childhood, with typical symptoms such as diarrhoea, weight loss and malabsorption (Young and Pringle, 1971). Eventually, however, it was also found in other age groups, including elderly people, albeit often with milder symptoms (Logan et al., 1983; Mäki et al., 1988; Vilppula et al., 2008). Today coeliac disease is seen more as a systemic disorder including diverse extraintestinal manifestations such as the skin disease dermatitis herpetiformis (Marks et al., 1966), bone-related disorders (Mustalahti et al., 1999; Stenson et al., 2005), hepatic diseases (Volta et al., 1998; Kaukinen et al., 2002), dental enamel defects (Aine et al., 1990), infertility (Farthing et al., 1982; Collin et al., 1996), malignant lymphomas (Harris et al., 1967; Viljamaa et al., 2006) and neurological symptoms (Hadjivassiliou et al., 1996; Luostarinen et al., 1999). On the other hand, the disorder can also appear without any symptoms (Mäki et al., 2003), being detected usually by screening of relatives of coeliac disease patients and other risk groups (Mäki et al., 1991). Coeliac disease has nowadays come to be considered not a rare disease but affecting approximately 1% of the population and actually increasing over time; according to very recent studies its prevalence is already around 2 % (Lohi et al., 2007; Vilppula et al., 2009; Walker et al., 2010).

2.2 Diagnosis

Coeliac disease is characterised by gluten-dependent small-intestinal mucosal destruction in the duodenum and the upper compartment of the jejunum**.** According to the original classification by Marsh (Marsh, 1992), the disease develops gradually from initial infiltration of intraepithelial lymphocytes (IEL) to shortening of the villous structure together with enlargement of crypts, finally into overt villous atrophy and crypt hyperplasia (Figure 4).

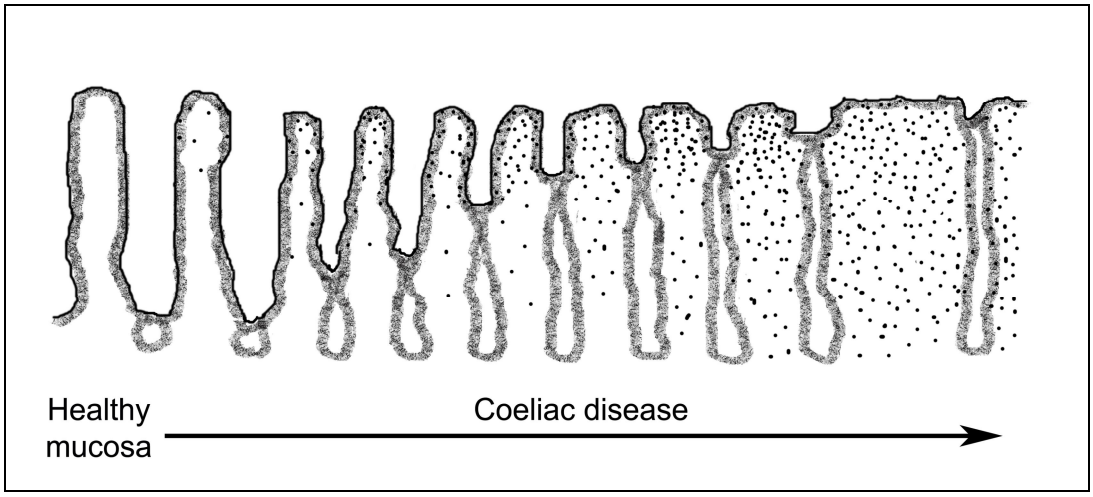

Figure 4. Gradual development of gluten-induced villous atrophy and crypt hyperplasia in coeliac disease over time. The figure is based on original publication (Marsh, 1992).

Other morphological and histological characteristics of the disease are decreased enterocyte cell height (Kuitunen et al., 1982) and increased numbers of IELs on the small-bowel mucosa (Ferguson and Murray, 1971; Marsh, 1992). The majority of the accumulated IELs between epithelial cells are CD3+ T cells expressing αβ-receptors on the cell surface, whereas a more striking feature of the disease is an increased density of CD3+ γδ+ IELs (Spencer et al., 1991; Kutlu et al., 1993). In addition, extensive accumulation of immune cells can be seen in the *lamina propria*, beneath the epithelium, the volume of the layer being thus some two times greater than in the healthy mucosa (Risdon and Keeling, 1974). However, with adherence to a strict gluten-free diet the morphology and inflammation in the mucosa slowly heals (Wahab et al., 2002).

Another important feature of the disease is the appearance of IgA-class autoantibodies, targeted against an endogenous enzyme, TG2 (Dieterich et al., 1997) and ingested gluten (Carswell and Ferguson, 1972). Of these, the first introduced diagnostic autoantibodies were circulating anti-reticulin and anti-gliadin antibodies (Seah et al., 1971; Carswell and Ferguson, 1972). Subsequently, detection of endomysial antibodies (EmA) became more common by reason of their superior sensitivity and specificity (Sulkanen et al., 1998a). The first EmA assay was based on detection of a typical reticular network pattern of staining in the monkey

oesophagus serving as an antigen (Chorzelski et al., 1983). However, the cost and ethical issues attending the method led to use of other tissues such as human umbilical cord (Ladinser et al., 1994). Since the observation that coeliac disease-specific antibodies are targeted against TG2 (Dieterich et al., 1997), a rapid and easily performed enzyme-linked immunosorbent assay (ELISA) has been successfully used in measuring TG2 autoantibodies (TG2-ab) from patients serum (Sulkanen et al., 1998b). Finally, the most recent diagnostical tool for autoantibody detection is an ELISA-based immunoassay where the autoantibodies bind to synthesised deamidated gliadin peptides (Schwertz et al., 2004). Nowadays the circulating EmA, TG2-ab and gliadin peptide antibodies are widely used as supportive markers for the disease diagnosis.

The autoantibodies, targeted against TG2, are produced locally in the small-intestinal mucosa (Picarelli et al., 1996; Marzari et al., 2001; Wahnschaffe et al., 2001). In addition to being measured in patient serum, IgA class autoantibodies can also be found extracellularly, deposited *in situ* in different tissues of patients suffering from coeliac disease (Jos et al., 1979; Karpati et al., 1988; Korponay-Szabo et al., 2000; Hadjivassiliou et al., 2006). In the healthy small-intestinal mucosa, IgA is located in plasma cells, in contrast to the diseased condition where the deposits can also be found extracellularly below the epithelial basement membrane and mucosal blood vessels, targeted against TG2 (Korponay-Szabo et al., 2004; Salmi et al., 2006b). Interestingly, the mucosal IgA deposits appear already in the early phases of the disease, even without any signs of villous atrophy or mucosal inflammation (Kaukinen et al., 2005; Salmi et al., 2006a) and even before being detected in the serum (Korponay-Szabo et al., 2004; Salmi et al., 2006b). After withdrawal of gluten from the diet, the autoantibodies disappear from the patient's circulation and small-bowel mucosa, usually within one year (Sulkanen et al., 1998b; Kaukinen et al., 2005; Koskinen et al., 2009).

2.3 Genetics

The development of coeliac disease is dependent not only on gluten, the environmental trigger, but also on a genetic factor. It is well known that coeliac disease clusters in families (Petronzelli et al., 1997), shown also as high concordance between monozygotic twins (Greco et al., 2002). The key genetic risk factors for coeliac disease are located in the major histocompatibility complex (MHC) region on chromosome six, where the primary association lies in the human leukocyte antigen (HLA) genes (Sollid et al., 1989). These genes encode HLA DQ2 and DQ8 molecules, among others involved in presenting antigens to T cells. Most patients (90-95%) carry the DQ2 heterodimer (DQA1*05/DQB1*02), whereas a minority (5-10%) are usually positive for the DQ8 heterodimer (DQA1*0301/DQB1*0302) (Sollid et al., 1989). However, approximately one third of the normal population are positive for HLA DQ2 or DQ8 without having the disease (Sollid et al., 1989; Polvi et al., 1996), which would implify the involvement of additional genes in the disease process. Extensive genome-wide screening approaches have been under investigation to find other genetic factors contributing to the disease (Zhong et al., 1996; Greco et al., 1998; Liu et al., 2002; van Heel et al., 2007; Hunt et al., 2008; Dubois et al., 2010). A strong association in chromosome 19, in an intron of the gene encoding the myosin 9B (MYO9B) molecule, was found in the Dutch population (Monsuur et al., 2005) but could not be unambiguously confirmed by others (Amundsen et al., 2006; Koskinen et al., 2008). It has been proposed that MYO9B might have a role in controlling cellular permeability. Also regions harboring genes coding for immune response components, for example chemokines, cytokines, interleukins and T cell activation, have been suggested to contribute to the genetic predisposition for coeliac disease (Djilali-Saiah et al., 1998; Greco et al., 1998; Liu et al., 2002; Haimila et al., 2004; van Heel et al., 2007; Hunt et al., 2008). However, thus far none of these genes has been confirmed to have an essential *in vivo* role in the pathogenesis of coeliac disease.

2.4 Pathogenesis

2.4.1 Gluten peptides as a trigger in coeliac disease

The common feature of the *Triticeae* cereal prolamins is the high amount of repetitive glutamine- and proline-rich sequences which are highly resistant to proteolytic degradation by human gastric, pancreatic and brush-border enzymes, even in healthy individuals (Hausch et al., 2002; Shan et al., 2002). This results in the presence of relatively large peptides which are thought to predispose recognition of the small-bowel mucosal immune system and thereby further to the development of coeliac disease. It has been estimated that dozens of different peptides in gliadins, hordeins and secalins might be involved in the disease process (Vader et al., 2002b; Shan et al., 2005). Some of those peptides have been identified as toxic, inducing early effects on the small-bowel mucosal epithelium (Sturgess et al., 1994; Maiuri et al., 2000; Maiuri et al., 2003), whereas others are rather immunogenic, responsible for the activation of T cell-mediated adaptive immunity in the mucosal *lamina propria* and the release of proinflammatory cytokines (van de Wal et al., 1998; Anderson et al., 2000; Arentz-Hansen et al., 2000; Shan et al., 2002; Mazzarella et al., 2003).

Soon after the discovery of gluten, it was noted that even very small oligopeptides can induce the symptoms characteristic for the disease (Bronstein et al., 1966). The original assumption was that alpha-gliadins may be the only toxic fraction involved in the activation of coeliac disease (Kendall et al., 1972). However, a variety of peptides have since been synthesized from the full 266 amino acid-long α-gliadin to evaluate the most important toxic sequences, and several peptides were found to induce harmful effects *in vivo* and *in vitro* in the small-intestinal mucosa of coeliac disease patients (Ciclitira et al., 1984; Howdle et al., 1984; de Ritis et al., 1988; Fluge et al., 1994; Shidrawi et al., 1995; Martucci et al., 2003). Most of these derive from the N-terminal region of α-gliadin peptides for example toxic peptides 31-43 (Maiuri et al., 1996; Picarelli et al., 1999) and 31-49 (de Ritis et al., 1988; Sturgess et al., 1994) as well as the immunodominant peptides

57-68 (Arentz-Hansen et al., 2000) and 56-88 (Shan et al., 2002) (Figure 5). Especially the 33-residue-long immunodominant peptide 33-mer is markedly stable in the conditions prevailing in the human gut, remaining intact even in the presence of digestive enzymes (Shan et al., 2002), and might thus be a particular potent activator of the immune system in patients susceptible to coeliac disease.

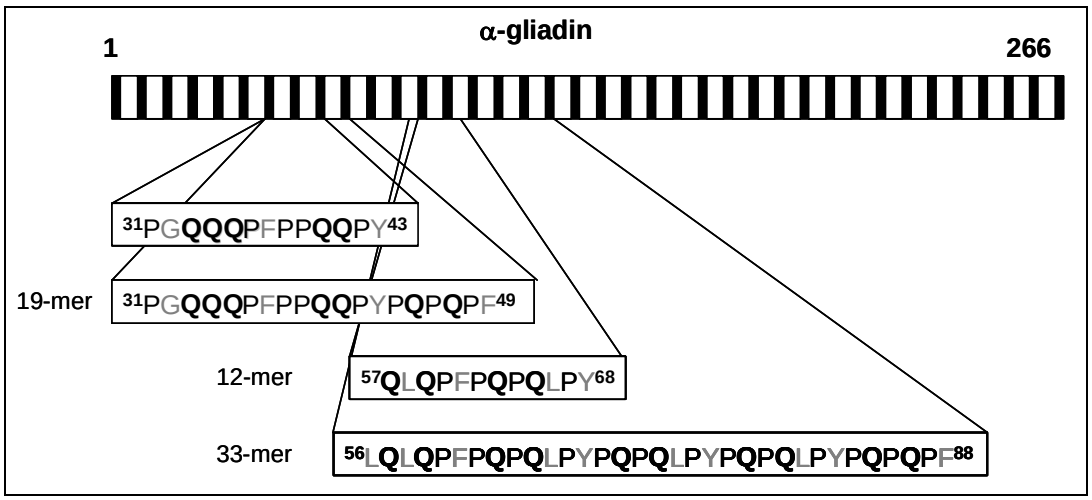

Figure 5. Immunostimulatory alpha-gliadin peptides of wheat, rich in proline (P) and glutamine (Q) residues. This figure is produced from the original publication (Shan 2005).

More recently similar glutamine- and proline-rich regions have also been found in γ- and ϖ-gliadins along with glutenins (Ciclitira et al., 1984; Howdle et al., 1984; van de Wal et al., 1999; Arentz-Hansen et al., 2002; Molberg et al., 2003; Shan et al., 2005; Dewar et al., 2006). In addition, extensive database searches have been applied to establish similarities between peptides of the *Triticeae* cereal prolamins (wheat, rye and barley) which might be involved in the disease mechanisms but differ from those found in non-toxic cereals such as oats, maize and rice (Kasarda et al., 1984; Shan et al., 2002; Vader et al., 2002a). Shan and co-workers (2005) identified several sequences known to be recognised by a specific population of T cells isolated from patients suffering from coeliac disease. Most were fairly long sequences, widespread within the α-gliadin, γ-gliadin, glutenin, hordein and secalin protein families, sharing similarities to the highly immunostimulatory 33-mer (Shan

et al., 2002; Shan et al., 2005). So far, however, none of these peptides has been specified as a sole trigger of coeliac disease.

2.4.2 Gluten peptides crossing the epithelium

The small-intestinal mucosal epithelial layer together with dendritic cells regulates molecular trafficking between the intestinal lumen and the submucosa, leading to either tolerance of or immunity to non-self antigens. Nevertheless, no specific coeliac disease-related receptors for gluten peptides have been reliably identified on the epithelial cells, although some preliminary studies have indicated that gliadin is recognised by a G protein-coupled receptor in the chemokine family CXCR3 on intestinal epithelial cells (Lammers et al., 2008). On the other hand it is thought that the peptides can pass the epithelial barrier by two different mechanisms: either by active transport via transcytosis (Zimmer et al., 1995; Matysiak-Budnik et al., 2003; Barone et al., 2007; Matysiak-Budnik et al., 2008; Schumann et al., 2008; Zimmer et al., 2009) or paracellularly through epithelial cell junctions (Figure 7) (Fasano et al., 2000; Matysiak-Budnik et al., 2003; Jabri and Sollid, 2009).

Enterocytes are capable of processing, transcytosing and presenting food antigens from the intestinal lumen to the T lymphocytes of the subjacent *lamina propria* (Hershberg and Mayer, 2000). It has been demonstrated that enterocytes are able to take up gliadin (Friis et al., 1992) and more specifically to carry peptides in association with MHC II antigens by transcytosis, allowing intact gluten peptides to enter the *lamina propria* (Zimmer et al., 1995; Zimmer et al., 2009). In another approach, the transferrin receptor CD71 has been shown to be responsible for transcytosis of gliadin peptides (Matysiak-Budnik et al., 2008).

Alterations in epithelial barrier function are often related to disease-inducing components (Groschwitz and Hogan, 2009). Clinical and experimental studies have indicated that the permeability of the epithelial cell layer is increased in coeliac disease (Bjarnason et al., 1983; Schulzke et al., 1995; Smecuol et al., 1997;

Clemente et al., 2003; Matysiak-Budnik et al., 2003; Pizzuti et al., 2004; Sander et al., 2005; Lammers et al., 2008), allowing gliadin to cross the intestinal barrier and activate the immune system. This phenomenon is related to modulation of epithelial cell junctions, of which the tight junctions have a major role in the regulation of paracellular transport, including uptake of nutrients, water and electrolytes as well as prevention of macromolecule leakage across the intestinal epithelium (Farquhar and Palade, 1963; Schneeberger and Lynch, 2004). Tight junctions form multiprotein complexes at the border between the apical and lateral epithelial membrane regions, participating in the regulation of cytoskeletal attachment, cell polarity, cell signaling and vesicle trafficking (Groschwitz and Hogan, 2009). They consist of integral transmembrane proteins, of which the most relevant are occludin (Furuse et al., 1993) and its interacting protein zonula occludens-1 (ZO-1) (Stevenson et al., 1986; Furuse et al., 1994), as well as claudins (Furuse et al., 1998; Turksen and Troy, 2004) and junctional adhesion molecules (JAMs) (Martin-Padura et al., 1998) (Figure 6).

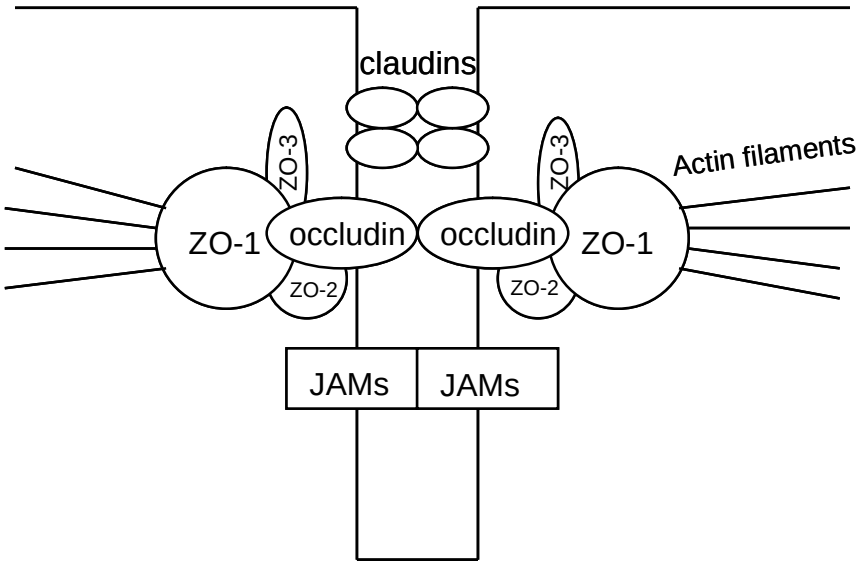

Figure 6. Schematic illustration of the tight junction complex between epithelial cells. ZO= zonula occludens protein, JAM= junctional adhesion molecule. The figure is drawn according to original publications (Dolfini et al., 2005a; Groschwitz and Hogan, 2009).

A number of investigations have suggested that the expression of the tight junction-associated proteins is abnormal in the small-bowel mucosa of coeliac disease patients (Schulzke et al., 1998; Montalto et al., 2002; Pizzuti et al., 2004; Szakal et al., 2010) and is directly affected *in vitro* by gliadin peptides on the epithelium cells (Dolfini et al., 2005a; Sander et al., 2005; Ciccocioppo et al., 2006). In addition, recent studies have revealed the function of the zonulin molecule, identified as prehaptoglobin-2, in the regulation of tight junctions. Although the exact role of the molecule is not known, it seems to contribute to a subsequent increase of intestinal permeability in coeliac disease (Fasano et al., 2000; Drago et al., 2006; Tripathi et al., 2009). It has also been established that the CXCR3 receptor is crucial for the release of zonulin, serving as a potential target receptor for gliadin binding on enterocytes (Lammers et al., 2008).

2.4.3 Activation of immune response

Innate immunity

Emerging evidence has implied that in coeliac disease leakage of gliadin peptides into the small-bowel mucosal *lamina propria* can stimulate epithelial cell damage directly without activation of CD4+ T cells (Figure 7) (Jabri et al., 2000; Maiuri et al., 2000; Maiuri et al., 2003; Sakly et al., 2006; Barone et al., 2007; Reinke et al., 2009) as well as activate monocytes and dendritic cells in the *lamina propria* (Jelinkova et al., 2004; Palova-Jelinkova et al., 2005; Raki et al., 2006).

One of the innate immunity components, interleukin-15 (IL-15), has been closely shown to be implicated in the pathogenesis of coeliac disease (Figure 7) (Jabri et al., 2000; Meresse et al., 2004; Benahmed et al., 2007; Yokoyama et al., 2009). IL-15 is over-expressed in both the intestinal epithelium and the *lamina propria* of patients with active coeliac disease (Maiuri et al., 2000; Maiuri et al., 2003; Mention et al., 2003; Di Sabatino et al., 2006). IL-15 is produced locally in intestinal epithelial cells, dendritic cells, macrophages and mononuclear cells after stimulus by stress

(Waldmann and Tagaya, 1999). It contributes to the stimulation of CD8+ IEL to differentiate into natural killer cells (Ebert, 1998; Meresse et al., 2004), which can participate directly in epithelial cell destruction via recognition of two important stress-induced proteins, MHC class I gene A and B (MICA and MICB) on the epithelial cells (Hue et al., 2004). MICA and MICB serve as ligands for natural killer cell group 2 member D receptors (NKG2D) expressed on the surface of CD8+ αβ+T cells, γδ+T cells and natural killer cells (Bauer et al., 1999). A number of studies have demonstrated that MICA and NKG2D receptors are over-expressed in coeliac disease (Jabri et al., 2000; Maiuri et al., 2001a; Hue et al., 2004; Meresse et al., 2004; Meresse et al., 2006) and after *in vitro* gliadin challenge (Hue et al., 2004; Martin-Pagola et al., 2004). Interestingly, the infiltrative CD8+ IELs (αβ+ T cells and γδ+ T cells) appear on the small-bowel mucosa already in the early phases of the disease process (Kutlu et al., 1993), and may thus play more than a secondary role in the pathogenesis of coeliac disease.

Adaptive immunity

Once gliadin peptides have crossed the epithelial barrier and entered the *lamina propria*, gliadin is thought to be deamidated by TG2 (Figure 7) (Molberg et al., 1998; van der Wal et al., 1998), thought, it is not known for sure where this reaction occurs. However, during the deamidation process, particular glutamine amino acids in gliadin peptides are modified to negatively charged glutamic acid residues (Vader et al., 2002a). The deamidation favors the interaction of gliadin peptides with HLA-DQ2 molecules on antigen-presenting cells (Sjöström et al., 1998; Arentz-Hansen et al., 2000). TG2 is expressed in many different human tissues and organs, being found both intracellularly and extracellularly. It belongs to the ubiquitious family of calcium-dependent transamidating enzymes, having diverse functions such as post-translational cross-linking of proteins, assembly of extracellular matrix components, mediation of transmembrane signaling and wound healing (Fesus and Piacentini, 2002). It has been noticed that TG2 activity is increased in coeliac disease during inflammation (Bruce et al., 1985; Esposito et al., 2003) but additional evidence is still needed to clarify the importance of this enzyme in the pathogenesis.

After deamidation, gliadin peptides are easily recognised by HLA DQ2 or DQ8 molecules on antigen-presenting cells (Figure 7), which in turn activate a specific population of CD4+ T cells (Molberg et al., 1998). T cells start to proliferate and accumulate in the mucosal *lamina propria*. By producing proinflammatory cytokines such as interferon-gamma (IFN-γ), the activated CD4+ T cells further provoke intraepithelial cytotoxic T cells to damage intestinal epithelial cells (Figure 7) (Nilsen et al., 1995). Activated T cells also induce B cells to differentiate into plasma cells, producing disease-specific IgA-class antibodies. It has been demonstrated that coeliac autoantibodies may contribute to intestinal barrier modulation by increasing the transcellular transport of gliadin through the epithelial barrier (Matysiak-Budnik et al., 2008), increasing epithelial cell permeability (Zanoni et al., 2006), inhibiting the differentiation of epithelial cells (Halttunen and Mäki, 1999) and stimulating the proliferation and actin reorganisation of intestinal epithelial cells (Barone et al., 2007).

It is evident that activation of adaptive immunity components is not in itself sufficient to explain all the phenomena characteristic of coeliac disease. There is increasing evidence of a more complex disease with both innate stress signals and T cell-mediated tissue destruction. In addition defective controlling of oral tolerance by regulatory T cells might be involved in the disease process. The regulatory T cells secrete cytokines such as transforming growth factor-β (TGF-β), interleukin-10 (IL-10) and interleukin-4 (IL-4), which in turn inhibit the induction of T cells (Izcue et al., 2009). Some preliminary evidence of impaired function of forkhead box P3 (FOXP3)-positive CD25+CD4+ regulatory T cells in coeliac disease has recently been presented (Figure 7) (Bhagat et al., 2008; Tiittanen et al., 2008; Granzotto et al.,2009).

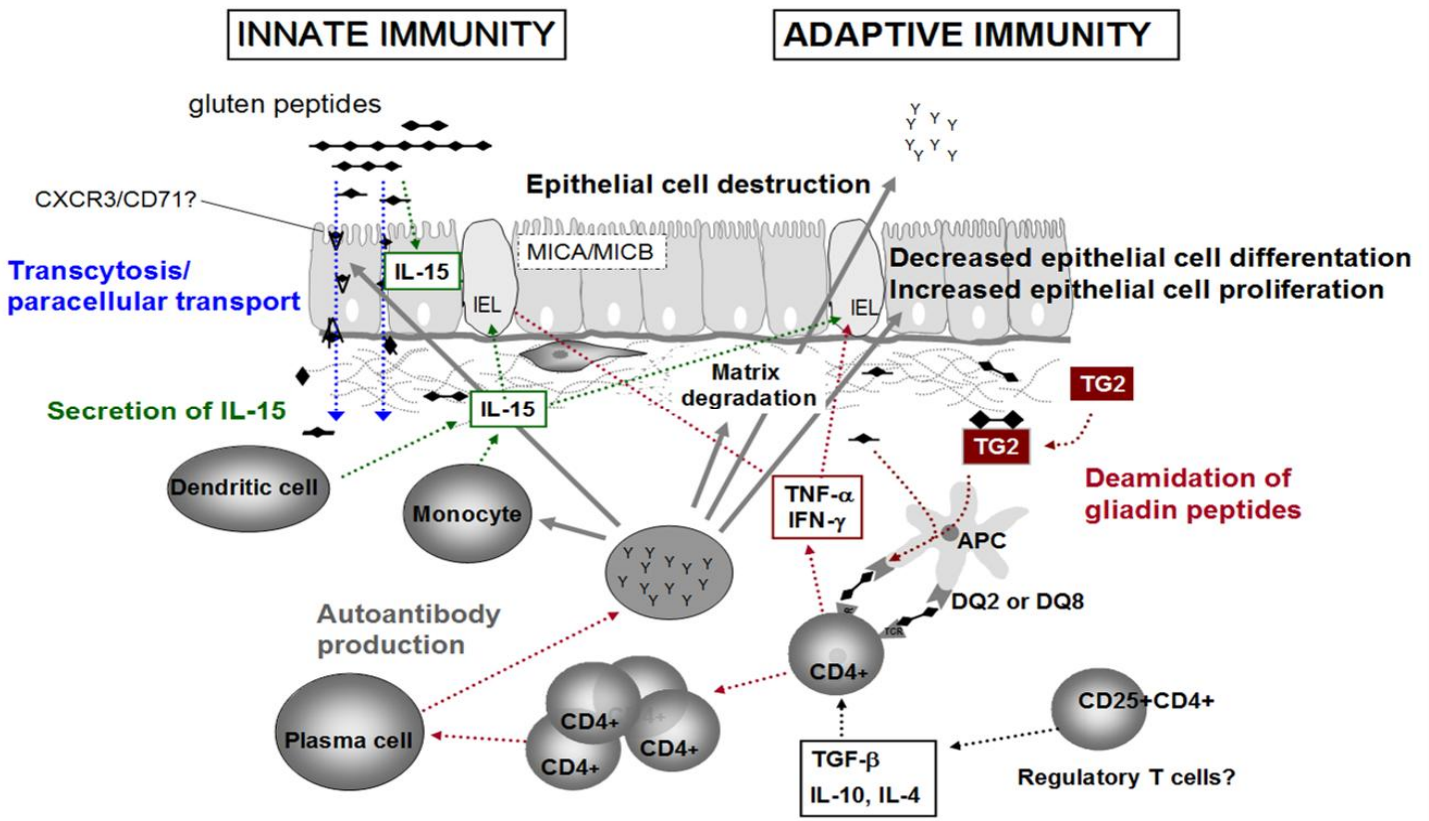

Figure 7. Summary of the pathogenesis of coeliac disease. CXCR3/CD71=receptors for gluten recognition; IEL=intraepithelial lymphocyte; APC=antigen presenting cell; IL=interleukin; MICA/MICB=MHC class 1 chain-related gene A/B; TNF-α= tumor necrocis factor-alpha; IFN-γ=interferon-gamma; TGF-β=transformin growth factor-beta; TG2=transglutaminase 2. This figure is produced from the original publication (Lindfors et al., 2009).

2.5 Treatment

2.5.1 Current approaches

At present, the only treatment for coeliac disease is a life-long, strict gluten-free diet, excluding all wheat-, rye- and barley-containing food products. However, several overwhelming problems attend the restricted diet. In many countries gluten-free products are poorly available, inadequately labelled and expensive, and the nutritional value is far from the recommended (Thompson et al., 2005). Moreover, some patients find the diet constricting during social events and travel. It is thus not surprising that compliance frequently remains inadequate (Hall et al., 2009), and further a minority of patients do not respond to a gluten-free diet (refractory coeliac disease) and are treated with harsh immunosuppressive drugs (Daum et al., 2005). In light of this, a search for alternative treatment is clearly warranted.

2.5.2 Future approaches

During recent years several lines of research aim to develop alternative therapies for coeliac disease. The majority of the studies in question have focused on reducing the disease-specific inflammation on the small-intestinal mucosa. Approaches include blocking of innate immunity or adaptive immunity components as well as the most relevant disease-associated autoantigen, TG2. Another prominent strategy is to eliminate gluten toxicity already before absorption into the small-bowel mucosa. There are in practice two different approaches in the detoxification of gluten peptides: either before gluten ingestion (food processing) or *in situ* during digestion in the gastrointestinal tract (medical treatment). Since the human digestive enzymes are insufficient to hydrolyse proline- and glutamine-rich gluten peptides, exogenous enzyme supplements have been designed to complete the cleavage of remaining harmful peptides (Shan et al., 2002). Exogenous enzyme therapy is in fact a fairly

old idea emerging after discussion as to whether coeliac disease is caused by a digestive enzyme deficiency. It was already demonstrated in 1964 that gluten could be degraded by using a papain enzyme (Messer et al., 1964). A summary of alternative treatment strategies for coeliac disease is given in Table 1.

Table 1. Alternative treatment strategies for coeliac disease.

Therapeutic strategy	**Status**	**Reference**
Genetic engineering of cereals	Preclinical	(Vader 2003; Spaenij-Dekking 2005 Pizzuti 2006; van den Broeck 2009)
Immunomodulation by gluten peptide analogues	Preclinical	(Biagi 1999; Senger 2003)
Transamidation of glidin	Preclinical	(Gianfrani 2007)
Vaccine	Phase I-II clinical trial	(Shan 2002; Anderson 2008; Keech 2009)
Tissue transglutaminase inhibitors	Preclinical	(Choi 2005; Siegel 2007)
Blocking of HLA-DQ presentation	Preclinical	(Xia 2007; Kapoerchan 2009)
Silencing of gluten-reactive T-cells	Preclinical	(Maurano 2001;Hue 2004; Meresse 2004)
Cytokine therapy (IL-10, IL-15, IFN-γ)	Phase I clinical trial	(Mulder 2001; Mention 2003; Hue 2004; Sollid and Khosla 2005; Salvati 2005; Yokoyama et al., 2009)
Interference with the host's immune response Hookworm *Necator americanus* infection	Phase II clinical trial	(Daveson 2009)
Inhibition of paracellular permeability	Phase II clinical trial	(Fasano 2000; Paterson 2007)
Detoxification of gluten		
Lactobacillus or probiotics	Preclinical	(Di Cagno 2004; 2008; De Angelis 2006; Gobetti 2007; Rizzello 2007; Lindfors 2008)
Enzyme supplements:	Phase II clinical trial	
Bacterial prolyl endopeptidases (PEP) *Flavobacterium meningosepticum* *Sphingomonas capsulate* *Myxococcus xanthus*		(Shan et al. 2002; 2004; Piper 2004; Gass 2005; Matysiak-Budnik 2005; Marti 2005; Pyle 2005; Stepniak 2006; Ehren 2008)
PEP from fungi *Aspergillus niger* (AN-PEP)		(Stepniak 2006; Mitea 2008)
EP-B2 from barley		(Bethune 2006; Gass 2006; Bethune 2008)
Enterococci and fungal proteases		(M'hir 2009)
Combination of proteases:	Phase II clinical trial	
EP-B2 from barley and PEP from *S. capsulata*		(Siegel 2006; Gass 2007; Tye-Din 2009)
Aspergillopepsin from *Aspergillus niger* and Dipeptidyl peptidase IV (DPPIV) from Aspergillus oryzae		(Ehren 2009)

Enzyme therapy

Bacteria have an advanced ability to process food proteins by means of a variety of specific proteases. A group of enzymes, the prolyl endopeptidases (PEP), have been isolated from different microbial species such as *Flavobacterium meningosepticum, Sphingomonas capsulata* and *Myxococcus xanthus* with a view to use as a potential enzymatic therapy for coeliac disease (Shan et al., 2002; Shan et al., 2004). The advantage of PEP lies in its capacity to cleave highly resistant proline residues in proteins which are otherwise incompletely degraded in the human digestive system (Hausch et al., 2002). Although PEP is also expressed in some human tissues (Polgar, 2002; Myöhänen et al., 2007), it does not contribute to the assimilation of dietary proteins in the human gut.

PEP derived from different species have shown facilitated hydrolysis of intact gluten peptides such as the highly immunodominant 33-mer peptide (Shan et al., 2002; Piper et al., 2004; Shan et al., 2004; Gass et al., 2005; Shan et al., 2005). Moreover, pre-treatment of selected gluten peptides with PEP from *Flavobacterium meningosepticum* has significantly reduced the immunogenicity of the peptides, as shown by patient-derived intestinal T cell proliferation assay (Marti et al., 2005). Similarly, in a preliminary clinical study the pretreatment of gluten with PEP has prevented the development of fat or carbohydrate malabsorption in the majority of coeliac disease patients after a gluten challenge (Pyle et al., 2005). It nonetheless remains questionable whether PEP is alone capable of complete detoxification of gluten. In a study by Matysiak-Budnik and associates (2005), high concentrations of PEP and a prolonged incubation time were needed for full breakdown of the immunostimulatory peptides. In addition, PEP is able to cleave only rather short peptides (Shan et al., 2004) and the optimum pH for the enzyme is around 7, indicating that PEP would not function ideally in the conditions prevailing in the stomach and might thus fail to complete the degradation before small-intestinal absorption of gluten (Shan et al., 2004; Stepniak et al., 2006). On the other hand, it has been reported that the activity and stability of PEP might be enhanced by protein engineering (Ehren et al., 2008).

In another approach, similar proteases with more enhanced degrading properties together with stability in acidic conditions were isolated from the fungus *Aspergillus niger* (AN-PEP) (Stepniak et al., 2006). In the study in question, the AN-PEP proved highly efficient in the degradation of diverse T cell epitopes from α- and γ-gliadin as well as LMW- and HMW-glutenins in a relatively short time. Moreover, AN-PEP pretreatment clearly reduced gluten-specific T cell proliferation *in vitro* (Stepniak 2006). Recently, the efficacy of AN-PEP in degrading a slice of bread and a whole gluten-containing meal were investigated in a model which mimics the conditions found in the gastrointestinal tract *in vivo* (Mitea et al., 2008). In that system, AN-PEP cleaved gluten peptides already in the stomach compartment so effectively that hardly any recognisable T cell epitopes were passed into the small intestine. While these results indicate that AN-PEP might constitute a potential candidate for gluten degradation, its efficacy *in vivo* needs to be further evaluated.

While PEP and AN-PEP are highly proline-specific, other enzymes are needed for the degradation of glutamine-rich residues in the gluten peptides. In light of this, a cysteine endoprotease B, isoform 2 from barley (EP-B2) was selected for further analysis. EP-B2 is one of the proteases responsible for the proteolysis of seed storage proteins during the germination of barley. In a study by Bethune and associates (2006), the function and stability of the enzyme were carefully evaluated. It was observed that the advantage of the enzyme lies in its stability and activity in the diverse pH ranges found in the gastrointestinal tract; however, the enzyme was seen to be susceptible to trypsin, which might lead to inactivation in the duodenum. In early proof-of-concept studies, EP-B2 showed efficient cleavage of the glutamine-rich α2-gliadin peptide and 33-mer (Bethune et al., 2006). Promising results have also been obtained in preliminary *in vivo* animal trials. In a rat model, oral administration of EP-B2 accelerated degradation of a gluten-containing meal already in a stomach compartment of the animals, indicating active function of the enzyme in gastric conditions (Gass et al., 2006). Similarly, EP-B2 has prevented a clinical response to gluten in gluten-sensitive rhesus macaques (Bethune et al., 2008).

Although all the above-mentioned enzymes can notably accelerate gluten degradation, recent studies have indicated that a combination of the enzymes might be superior in the full breakdown of gluten peptides (Siegel et al., 2006; Gass et al., 2007). For example, EP-B2 would hydrolyse intact gluten proteins, whereafter PEP is able to cleave proline residues from remaining (still inflammatory) peptide fragments (Gass et al., 2007). Co-administration of EP-B2 with PEP either from *Sphingomonas capsulata* or *Flavobacterium meningosepticum* has led to enhanced degradation of both immunostimulatory gluten peptides and whole-wheat bread (Siegel et al., 2006; Gass et al., 2007). In addition, in the studies in question, activation of T cell proliferation was abolished after such pretreatment. Another combination of enzymes was introduced by Ehren and associates (2009), who used Aspergillopepsin from *Aspergillus niger* and dipeptidyl peptidase IV (DPPIV) from *Aspergillus oryzae*. These two enzymes markedly enhanced gluten digestion similarly to EP-B2, although neither alone was able to cleave immunostimulatory gluten peptides.

Finally, some of the proteases of pharmacological interest have already been presented in preliminary clinical trials (Pyle et al., 2005; Tye-Din et al., 2009). In the last mentioned study (Tye-Din et al., 2009), oral administration of an enzymatically pre-treated gluten meal showed a protective role of the drug candidate in a majority of the coeliac disease patients. The enzyme combination used in the study was EP-B2 from barley and PEP from *Sphingomonas capsulata*.

3. *In vitro* models for gluten toxicity

One of the most conspicuons limitations in the field of coeliac disease research is the lack of a functional disease-specific animal model. A number of attempts have been made to create a mouse model for gluten-dependent enteropathy characterised by the coeliac disease-specific association with the HLA-DQ2 or DQ8 molecules, small-bowel mucosal damage and autoantibody production (Smart et al., 1992; Marietta et al., 2004; Di Niro et al., 2008; Verdu et al., 2008; de Kauwe et al., 2009; Freitag et al., 2009). So far, however, none of these attempts has yielded a mouse phenotype which would fully correlate with the disease in humans. For example transgenic mice expressing genetically the human HLA-DQ8 molecules developed blistering pathology and IgA deposits into skin similar to that seen in dermatitis herpetiformis (Marietta et al., 2004). However, no small-intestinal mucosal villous atrophy or crypt hyperplasia typical for coeliac disease appeared in mice (Marietta et al., 2004; Verdu et al., 2008; de Kauwe et al., 2009). Recently, coeliac patient derived anti-TG2 antibodies were shown to cause intensive brain ataxia in mice (Boscolo et al., 2010). But injection of either conventional anti-gliadin antibodies (Smart et al., 1992) or anti-TG2 antibodies (Di Niro et al., 2008) did not mediate pathological changes in the small intestine of mice. In contrast, gluten-dependent small-intestinal damage was achieved after transfer of gliadin-specific T cells into mice but the phenotype presented rather duodenitis occurring in Crohn's disease than coeliac disease (Freitag et al., 2009).

Transferring of coeliac disease into mice, especially with the fenotype characterized by all the diverse symptoms seen in humas is still problematical. Nevertheless, animal models are useful for example when studying the efficacy of a potential drug candidate in a disease of interest (Gass et al., 2006; Bethune et al., 2008). In those cases animal models offer investigation of an overall condition superior to single cell or tissue culture. However, since some of the biological mechanisms differ greatly from animals to humans, investigation of a particular

cellular or molecular mechanism is probably still more reliably presented in *in vitro* models, originated directly from human.

3.1 Epithelial cell culture models

The epithelium of the small-intestinal mucosa constitutes a highly dynamic system with especially rapid regeneration of cells from the crypts towards the villous tips through migration, differentiation and apoptosis. Different two- or three-dimensional intestinal cancer cell lines have been widely applied in studying the function of the small-bowel mucosa in the context of coeliac disease (Table 2). Of these, a colorectal adenocarcinoma cell line, Caco-2 cells spontaneously undergo differentiation in culture conditions, forming a confluent, polarised monolayer with an apical brush border and tight junctions (Bolte et al., 1998). Another colon carcinoma cell line, T84, derived from a lung metastasis, is non-polarised when cultured alone on plastic but differentiates into a two-to-three-fold polarised layer when cultured on collagen (Madara et al., 1987). These cells also express tight junctions and apical microvilli.

Table 2. Effects of wheat gliadin on intestinal epithelial cells *in vitro*.

Target	**Function**	**Cell line**	**Reference**
Growth	Changes in cell shape and size	Human embryonic intestinal cells	Hudson 1976
	Reduced viability of cells	RMC-5 (human embryo) and Hep-2 (larynx carcinoma)	Rocca 1983
	Reduced cell growth	Lovo (human colon carcinoma), Caco-2	Giovannini 1995; Dolfini 2002; 2003; 2005; Sakly 2006
	Increased proliferation	Caco-2 (human colon adenocarcinoma)	Barone 2007
	Increased apoptosis	Caco-2, T84 (human colon carcinoma)	Giovannini 2000; 2003; Maiuri 2001;2003; 2005
Cytoskeleton	Reduction of F-actin	Intestine 407 (Human embryonic intestinal cells)	Sjolander 1988
	Reorganisation of F-actin and tight junctions	IEC-6 (rat intestinal), Lovo, Caco-2	Clemente 2003; Pizzuti 2004; Dolfini 2005b; Sander 2005; Maiuri 2005; Ciccocioppo 2006; Drago 2006; Barone 2007; Reinke 2009
	Increased cell layer permeability	IEC6, Caco-2	Sander 2005; Lammers 2008
Agglutination	Increased agglutination	K562 (chronic myelogenous leukemia)	Auricchio 1984; Dessi 1992; De Vincenzi 1995
Cell metabolism	inhibition of DNA and RNA synthesis	Caco-2	Giovannini 1996
Oxidative stress	Increased lipid peroxidation	Caco-2	Rivabene 1999
	Reduced GSH-related enzyme activity	Lovo	Dolfini 2002; 2005a

3.2 Patient-derived T-cell lines and clones

Extensive evidence indicating that CD4+ T cells, associated with recognition of HLA DQ2 and DQ8 molecules, are strongly implicated in the pathogenesis of coeliac disease has inspired researchers to develop *in vitro* T cell models. Coeliac disease-specific T cells raised against gluten have been obtained both from the small-intestinal mucosa (Lundin et al., 1993; van de Wal et al., 1998; Ellis et al., 2003) and from the peripheral blood (Gjertsen et al., 1994; Nilsen et al., 1996; Anderson et al., 2005; Raki et al., 2007) of patients suffering from coeliac disease. HLA-DQ2- or DQ8-restricted T cells isolated from small-bowel mucosal biopsies are typically cultured *in vitro* in the presence of stimulating gluten peptides (T cell lines) whereafter single, gluten-reactive monoclonal cells (T cell clones) can be further isolated and maintained in culture conditions. In contrast, peripheral blood mononuclear cells are isolated from treated coeliac disease patient serum after a short-term *in vivo* gluten challenge (Anderson et al., 2000). In both cases, activation of T cells is typically reflected in an increase in cell proliferation and INF-γ-predominant cytokine production (Nilsen et al., 1996; Ellis et al., 2003; Anderson et al., 2005).

Most gut-derived T cells do not recognise native gluten peptides unless they are deamidated by TG2 (Molberg et al., 1998; Sjöström et al., 1998), a phenomenon thought also to occur in *in vivo* conditions. The majority of studies have concentrated on the identification of gliadin epitopes such as the diverse epitopes found in immunodominant α-gliadin 33-mer (Shan et al., 2002; Qiao et al., 2004). Furthermore, some TCL and TCC also recognise deamidated secalin and hordein peptides (Vader et al., 2003), being thus especially useful in the systematic characterisation of the T cell-reactive gluten epitopes of coeliac disease-related cereals (Arentz-Hansen et al., 2002; Beissbarth et al., 2005; Shan et al., 2005). However, the general problem with these cells is that coeliac patients seem to respond to distinct gluten peptides (Lundin et al., 1997; Vader et al., 2002b), this making investigations of the overall toxicity of different peptides a challenging task. To overcome this problem, use of several simultaneous T cell lines from different patients is probably needed.

3.3 Small-intestinal mucosal biopsies

Culturing of small-intestinal mucosal biopsies derived *ex vivo* from coeliac disease patients was originally introduced by Browning and Trier (1969). The samples can be maintained viable in an organ culture system for 24-48 hours (Jos et al., 1975) or even longer, although there is no guarantee for the quality of mucosal morphology after a long-term culture (Falchuk et al., 1974). In preliminary studies, the organ culture method was used in a manner opposite to that currently applied. Culture conditions were thought to mimic a gluten-free diet, so that the small-bowel mucosal morphology would improve after gluten was excluded from the culture (Falchuk et al., 1974; Jos et al., 1975; Hauri et al., 1978; Fluge and Aksnes, 1981). In contrast, the present organ culture system is generated to illustrate the harmful effects of gluten added to the culture supernatant. Since the *ex vivo* biopsy culture contains a wide variety of cell types expressed in the intestinal mucosa *in vivo*, the method allows researchers to investigate an extensive range of markers for gluten toxicity, from the epithelium to activation of immune components in the mucosal *lamina propria* (Table 3). One particular benefit of the method derives from in the fact that various features characteristic for the disorder can be reproduced from biopsies of treated coeliac disease patients, thus allowing researchers to find mechanisms related to the development of coeliac disease from healthy mucosa to the active phase of the disease. In addition, some researchers have suggested the organ culture method even as a potential tool for coeliac disease diagnosis (Bonamico et al., 2005; Picarelli et al., 2006).

However, in measuring autoantibody production in cultured biopsy samples in response to gliadin, results have been rather contradictory. It has been demonstrated that gliadin stimulates EmA secretion in biopsies from untreated and treated coeliac disease patients (Picarelli et al., 1996; Picarelli et al., 1999; Picarelli et al., 2001; Bonamico et al., 2005; Picarelli et al., 2006). Other studies, to the contrary, have not been able to repeat the results, EmA being produced in only half of the cases (Biagi et al., 2000; Carroccio et al., 2002) or couldn’t be stimulated at all (Vogelsang et al., 1999) in biopsies from treated coeliac disease patients.

Table 3. Effects of gliadin on small-intestinal mucosal biopsies from coeliac disease patients *ex vivo*.

Target	Function	Reference
Growth	Increased epithelial cell proliferation	Maiuri 2000
	Increased epithelial cell apoptosis	Maiuri 1998; 2000; 2003; 2005
	Increased Fas expression related to apoptosis	Mazzarella 2003; 2008; Salvati 2005
Epithelium	Decreased epithelial cell height	Howdle 1981; Simpson 1983; de Ritis 1988; Fluge 1981; 1994; Walz 1996; Shidrawi 1995; Biagi 2000; Martucci 2003; Pizzuti 2006
	Actin reorganization	Barone 2007: Maiuri 2005
	Induce HLA-DR expression	Fais 1992; Maiuri 1996; 2003; Pizzuti 2006
	Induce ICAM-1 expression	Maiuri 1998; 2003
Innate immunity	Increased expression of IL-15	Maiuri 2000; 2003
	Increased expression of IL-21	Fina 2007
	Enhanced expression of MICA/MICB	Hue 2004; Martin-Pagola 2004
	Increased amount of CD8+ T cells	Maiuri 2001
	Increased CD8+CD25+ T cells	Mazzarella 2008
Adaptive immunity	Increased number of CD3+, CD25+ and CD80+ T cells	Maiuri 1996; 1998; 2001; 2003; Salvati 2005; Pizzuti 2006 Mazzarella 2008
	Upregulation of IFN-γ mRNA or protein	Salvati 2005; Maiuri 1998; 2005; Pizzuti 2006
	Secretion of IFN-γ	Fina 2007
	Increased CD83+ cells	Maiuri 2003
Autoantibody production	Endomysial antibodies	Picarelli 1996; 1999;2001; 2006; Maiuri 1998 Biagi 2000; Carroccio 2002; 2006
	IgA, IgM and anti-gliadin antibodies	Ciclitira 1986
	Tissue transglutaminase antibodies	Carroccio 2006
Other	Upregulation of TG2	Maiuri 2005
	Increased alkaline phosphatase activity	Falchuk 1974; 1978
	Increased cyclo-oxygenase (COX-2) positive cells	Maiuri 2003
	Increased MAP-kinase activity	Maiuri 2003

Fas=transmembrane protein related to apoptosis; HLA-DR=human leukocyte antigen; ICAM-1=intercellular adhesion molecule 1; IL=interleukin; MICA/MICB=MHC class 1 chain-related gene A/gene B; IFN-g=interferon gamma; IgA=immunoglobin A; TG2=transglutaminase 2; COX2=cyclo-oxygenase 2; MAP=mitogen-activated protein

It has since been debaved whether autoantibody secretion is totally independent of gliadin challenge (Vogelsang et al., 1999). In addition, in some studies administration of gliadin proved unable even to change the morphology or histology of the small-intestinal mucosa (Hauri et al., 1978). Since these observations, there has been intensive discussion as to whether the organ culture method is a relevant tool at all as a model for gluten toxicity in coeliac disease.

AIMS OF THE STUDY

The purpose of the present study was to evaluate the use of different *in vitro* models for gluten toxicity in order to demonstrate the harmfulness of wheat gliadin and rye secalin in the context of coeliac disease. Futher, gliadin and secalin were hydrolysed with germinating cereal enzymes with an eye to developing novel treatment modes for the disease or improving the quality of coeliac-safe food products in the future.

The specific objectives were:

1. to evaluate the use of different *in vitro* models in coeliac disease research (**I**, **II**, **III**).
2. to evaluate the relevance of the human small-intestinal mucosal organ culture method as a model for gluten toxicity in coeliac disease **(I)**.
3. to compare the *in vitro* toxicity of rye secalin and wheat gliadin in a human intestinal epithelial cell culture **(III)**.
4. to detoxify gliadin and secalin with a variety of germinating cereal enzymes, evolutionarily selected for total cleavage of cereal storage proteins **(II, III)**.

4. MATERIALS AND METHODS

4.1 Preparation of gluten prolamines

The gliadin used here was isolated from wheat (Raisio Oy, Raisio, Finland) and secalin from rye flour (Myllyn Paras Oy, Hyvinkää, Finland). Both were dissolved in 1M NaCl, after which the mixtures were shaken and centrifuged at 4000 g for 20 min. The prolamins were then resuspended in water, centrifuged again and incubated with 70 % ethanol at 60ºC until totally dissolved. Thereafter, supernatants were collected and vacuum dried by centrifugation in order to isolate pure gliadin and secalin fractions. To mimic the conditions found in the human gastrointestinal tract, the prolamins were further dissolved in Na-acetate buffer, pH 4.0, and incubated with 3 mg of pepsin (P-6887, Sigma-Aldrich, Seelze, Germany) at 37ºC for 2 h, whereafter pH was adjusted to 7.0 together with addition of 3 mg of trypsin (T-7418, Sigma-Aldrich), and the mixture was incubated as above. Prepared gliadin and secalin fractions were then frozen and lyophilised.

Bovine serum albumin (BSA, A8806, Sigma-Aldrich) served as a negative control for peptic-tryptic (PT) treatment and was processed similarly to gliadin and secalin.

4.2 Pre-treatment with germinating cereal enzymes

Germinating cereal enzymes were isolated from three different cereal seeds: oats, wheat and barley. The seeds were germinated in a pilot malting apparatus (Joe White Malting System, Melbourne, Australia) at 14ºC until the most efficient activation of germinating cereal enzymes was achieved at day 8. The germinated grains were then homogenised in 50mM Na-acetate buffer, pH 5.0, whereafter the

water-soluable part was extracted by shaking at 4ºC for 1 h and centrifugation at 10 000g for 20 min.

Enzymatic pre-treatment of gliadin and secalin was effected by redissolving different concentrations (1-6 mg/ml) of these prolamins in 50 mM Na-acetate buffer, pH 4.0, together with germinating enzymes from oats, wheat or barley (0.1-100 µg/ml), and incubating the mixture at 37ºC for 24 h. The samples were then treated with pepsin and trypsin as above.

4.3 Analysis of prolamin degradation

4.3.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

After pre-treatment of gliadin and secalin with germinating cereal enzymes (see above), the reaction was stopped by heating (>95˚C, 10 min), whereafter 2 ml of intact prolamin and degraded prolamin products were subjected to SDS-PAGE analysis on 12% gel.

4.3.2 High-performance liquid chromatography and mass spectrometry (HPLC-MS)

The efficacy of germinating cereal enzymes to hydrolyse gliadin and secalin was assessed according to the molecular mass of degraded prolamin products, tandem mass spectrometry (MS/MS) and by comparing degradation products with the retention times of the highly stable standard α-gliadin peptides 12-mer (New England Peptide, Fitchburg, MA, USA) and 33-mer (New England Peptide) (Figure 5). The degradation process was analysed using an LTQ quadrupole ion trap mass spectrometer connected to a Surveyor MS HPLC system (Finnigan, San Jose, CA, USA), where the samples were separated in an XTerra C-8 column (20 mm x 2.1 mm, 3.5 mm particle) with a 2-64% acetonitrile gradient in acidic conditions over 46 min.

In addition, the efficacy of germinating oats, wheat and barley enzymes to cleave prolamins was compared by incubating different concentrations (0.1-100 µg/ml) of the enzymes with intact gliadin and secalin. The disappearance of mass-to-charge (m/z) signals from intact prolamins was plotted against enzyme concentrations. The results were drawn in curves, illustrating the half-maximal effective concentrations (EC_{50}) which gives an approximation of the enzyme concentration needed to reduce tha amount of each prolamin by 50%.

4.4 Cell cultures

4.4.1 Intestinal epithelial cells

Caco-2 cells (HTB-37, passage 19-81, American Type Culture Collection, Rockville, MD, USA) were grown under standard cell culture conditions until used in experiments. Prior to each experiment, the culture medium was replaced with starvation media containing Minimum Essential medium (GIBCO Invitrogen, Paisley, UK), 1% foetal bovine serum (GIBCO Invitrogen), 50 U/ml penicillin-streptomycin (GIBCO Invitrogen), 1 mM sodium pyruvate (Sigma-Aldrich), 1.5 g/l sodium bicarbonate (GIBCO Invitrogen) and 0.1 mM non-essential amino acids (GIBCO Invitrogen).

4.5 Epithelial cell culture experiments

In these experiments, cells were supplemented with the following study compounds (Table 4), each at a concentration of 1 mg/ml.

Table 4. Details of the study compounds used.

Supplementation	**Abbreviation**
None	Medium only
Pepsin and trypsin-treated bovine serum albumin	PT-BSA
Pepsin and trypsin-treated gliadin	PT-G
Germinating cereal enzymes pre-treated PT-gliadin	PT-G + enzymes
Pepsin and trypsin-treated secalin	PT-S
Germinating cereal enzymes pre-treated PT-secalin	PT-S + enzymes

Epithelial barrier function

To study epithelial cell layer permeability, cells were grown on Millicel Culture Plate Inserts (Millipore Corporate, Billerica, MA, USA) until reaching confluency (1000 ohms), measured as transepithelial resistance (TER) between cell monolayer (Millicell-ERS volt-ohm meter, Millipore Corporate). The cells were then exposed to the above-mentioned study compounds and TER was measured immediately and once every hour until reaching baseline level. The experiment was performed in duplicate and repeated three independent times.

Immunofluorescence stainings

The expressions of tight junctional protein occludin and ZO-1, related to epithelial barrier function, were studied in a confluent cell monolayer, cultured on Transwell polyester membrane inserts (Millipore Corporation) in the presence of the above-mentioned study compounds. After 24 h *in vitro* gluten challenge, the cells were fixed with 4 % paraformaldehyde (Merck), permeabilised with 0.1% Triton-X-100 (Sigma-Aldrich) and incubated with mouse monoclonal anti-occludin antibody (1:100, ZYMED, San Francisco, CA, USA) or mouse anti-ZO-1 antibody (1:100, ZYMED) for 60 min. Alexa Fluor 568 goat anti-mouse IgG (1:1000, Invitrogen, Carlsbad, CA, USA), incubated for 30 min, served as secondary antibody. All

stainings were visualised with an Olympus BX60 microscope (Olympus, Hamburg, Germany) and the experiments were performed three independent times.

When investigating actin cytoskeleton reorganisation, measured as actin membrane ruffling, cells were grown on eight-chamber glass slides (BD Biosciences, Erembodegem, Belgium) for 3-5 days, and thereeafter administered to the study compounds overnight. The cells were then treated as above and incubated with phalloidin–fluorescein isothiocyanate (FITC, 1:300, Sigma-Aldrich) for 15 min. In each sample, the membrane ruffle formation was calculated from at least 10 different cell clusters as a percentage of the total length of the cell cluster (analySIS software, Olympus Soft Imaging System GmbH, Munster, Germany).

Secretion of interleukin-15

The cells were cultured until reaching confluency, whereafter the study compounds were added and incubated for 24 or 48 h. Supernatants were then collected and used immediately for IL-15 sensitive ELISA (R&D Systems, Minneapolis, USA), according to manufacturer's instructions.

4.5.1 Patient-derived T cell clones and lines

The T cell-stimulatory ability of PT-BSA, PT-gliadin and enzymatically pre-treated PT-gliadin were tested in an *in vitro* T cell proliferation assay. The CD4+ T cells were isolated from the small-bowel mucosa of Norwegian coeliac disease patients as described elsewhere (Molberg et al., 2000). Since most of T cell clones and lines do not recognise native gluten peptides, the samples were first incubated with recombinant human TG2 (100 mg/ml) in the presence of 5 mM Ca^{2+} at 37˚C for 2 h.

Two different HLA DQ2-restricted TCC and two TCL were used in this study (Table 5). A HLA-DQ2 homozygous Epstein-Barr virus-transformed B lymphoblastoid cell line isolated from a coeliac disease patient was used for

antigen-presenting cells. Fifty thousand B cells were irradiated (100 Gy) and incubated at 37˚C overnight on a 96-well plate in RPMI 1640-medium supplemented with 10% human serum. Forty thousand gluten-specific T cells were then added to each well. T cell proliferation was measured by the uptake of 3H-thymidine (1 mCi/well, Hartmann Analytic, Braunschweig, Germany) added 20 h prior to harvesting. The cells were automatically harvested (Mach III, TomTec, Hamden, CT, USA) after 72 h onto glass fibre paper and 3H-thymidine incorporation was detected by liquid scintillation counting (Wallac MicroBeta TriLux 1450, PerkinElmer, Wellesley, MA, USA).

Table 5. T cell clones and lines used in the current study.

T cell clone or line	Epitope	Sequence	TG2 deamidation
TCC 436.5.6	DQ2-α-II epitope	PQPELPYPQ	Recognise only deamidated peptides
TCC 387.19	DQ2-γ-VII epitope	QQPEQPFPQ	Recognise only deamidated peptides
TCL 496.1.2	DQ2-γ-II epitope	IQPQ/EQPAQL	Recognise native and deamidated peptides
TCL KT.CD.E3	33-mer	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	Recognise only deamidated peptides
	DQ2-g-IV	SQPEQEFPQ	Recognise only deamidated peptides
	DQ2-g-VI	QQPFPEQPQ	Recognise only deamidated peptides

TCC=T cell clone; TCL=T cell line

4.6 Small-bowel mucosal biopsies *ex vivo*

4.6.1 Patients

Small-bowel mucosal biopsies were taken upon upper gastrointestinal endoscopy from untreated and treated coealic disease patients and from non-coeliac controls (Table 6). The diagnosis of all coeliac disease cases enrolled in the studies was confirmed according to the European Society of Pediatric Gastroenterology and Nutrition criteria (Walker-Smith et al., 1990). Moreover, HLA-typing was performed in each case. Genetic analyses were made at the Department of Tissue Typing, Finnish Red Cross Blood Service, Helsinki and the Department of Molecular Genetics of Immunological Diseases, University of Helsinki, Finland using the DELFIA® Celiac Disease Hybridization Assay (PerkinElmer Life and Analytic Sciences, Wallac Oy, Turku, Finland). The study protocol was approved by the Ethical Committee of Tampere University Hospital, Tampere, Finland and informed consent was obtained from each participant.

Table 6. Details of the patients participating in the studies (**I**,**II**).

Study I	**n**	**Patients**	**Median age**	**Females**	**Gluten-free diet**	**Histology**	**Serum EmA**	**Serum TG2-ab (U)**	**HLA-typing**
	5	Untreated coeliac disease	48 years (range 43-71 years)	5/5	No	Subtotal villous atrophy with crypt hyperplasia	1:500 (median) (range 1:5-1:4000)	58.1 (median) (range 4.7-100)	DQ2/DQ2+DQ8 5/5
	20	Treated coeliac disease	54 years (range 23-73 years)	16/20	At least 1 year (range 1-20 years)	Normal small-bowel mucosal morphology	negative	negative	DQ2/DQ2+DQ8 20/20
	6	Non-coeliac controls	53 years (range 24-70 years)	3/6	No	Normal small-bowel mucosal morphology	negative	negative	nd

Study II	**n**	**Patients**	**Median age**	**Females**	**Gluten-free diet**	**Histology**	**Serum EmA**	**Serum TG2-ab**	**HLA-typing**
	22	Untreated coeliac disease	44 years (range 17-74 years)	14/22	No	Subtotal villous atrophy with crypt hyperplasia	1:100 (median) (Range 0-1:500)	17.4 (median) (Range 0-64)	DQ2/DQ2+DQ8
	6	Non-coeliac controls	53 years (range 18-68 years)	3/6	No	Normal small-bowel mucosal morphology	negative	negative	nd

n=number of patients; nd=not determined; U=unit

4.6.2 Organ culture

Altogether seven duodenal biopsies were taken from each patient. Two of them were immediately snap-frozen in liquid nitrogen with optimal cutting temperature compound (OCT, Tissue-Tek, Sakura Finetek Europe, Alphen aan den Rijn, Holland) and used in standard morphology analyses for diagnostic purposes. Three samples were transferred to the laboratory on ice and cultured on the organ culture system at +37ºC for 24 or 48 h depending on further analyses (Figure 8). The samples were placed villi upwards on a sterile stainless steel grid positioned over the medium in a central well of the organ culture dish (Falcon, Becton Dickinson and Co, NJ, USA) and cultured in RPMI-1640 medium (GIBCO Invtirogen) containing 15 % heat-inactivated foetal bovine serum (GIBCO Invtirogen), 100 µg/ml streptomycin (GIBCO Invtirogen), 100 U/ml penicillin (GIBCO Invtirogen), 4 mM L-glutamine (GIBCO Invtirogen), 50 µg/ml insulin (Sigma-Aldrich) and 10 mM HEPES buffer (GIBCO Invitirogen). After culture, supernatants were collected and stored at -70ºC. Biopsy samples were washed with 0.9 % NaCl, and free fluid removed after which they were snap-frozen with OCT and stored at -20ºC until processed for stainings. For the experiments, biopsies were cut into 5-µm-thick sections on glass slides.

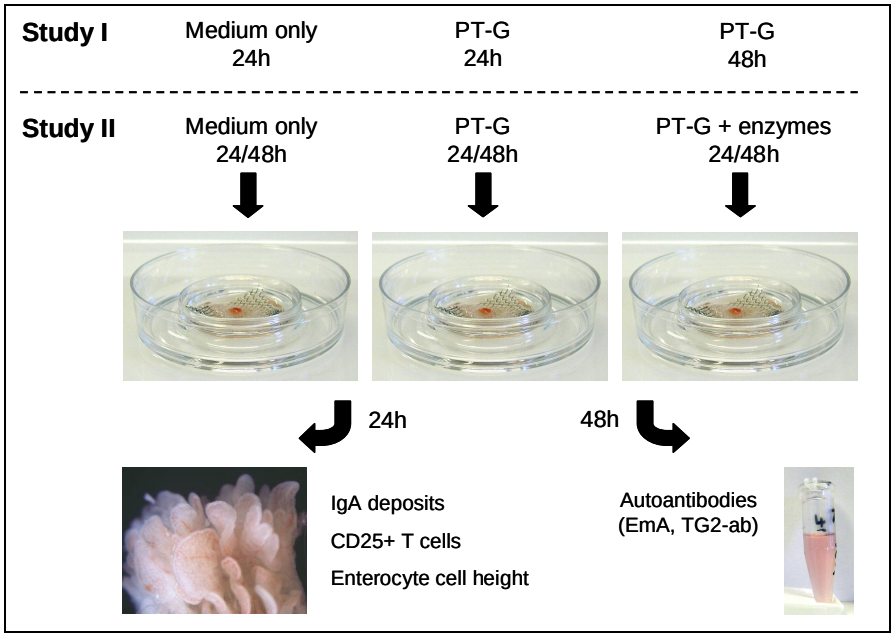

Figure 8. Human small-intestinal mucosal organ culture method.

4.6.3 Organ culture experiments

Endomysial antibodies

EmA was measured from patients' serum and undiluted organ culture supernatants using an indirect immunofluorescence assay. Samples were incubated on 7-µm-thick human umbilical cord sections used as antigen for 30 min. The sections were then washed and incubated with FITC-conjugated anti-human IgA (Dako A/S, Glostrup, Denmark) for 30 min. Stainings were analysed blindly using Olympus BX60 microscope. A serum dilution of 1:≥5 was considered positive. The antibody titres for organ culture supernatants were estimated as follows: negative (neg), weak positive (+), strong positive (++, +++ or ++++).

Transglutaminase 2 autoantibodies

TG2-ab were detected in culture supernatants using an ELISA method (Celikey®, Phadia, Freiburg, Germany) according to manufacturer's instructions. Culture supernatants were used undiluted, whereas serum samples were diluted 1:100. A unit value (U) ≥5 was considered positive in both serum and supernatant samples.

Small-bowel mucosal TG2-specific IgA deposits

IgA deposits were stained from the small-bowel mucosal biopsy sections using an immunofluorecence method. The biopsy specimens were treated with mouse monoclonal anti-transglutaminase antibody (1:200, Clone CUB 7402, Mouse Mab IgG1/kappa, Labvision, Fremont, CA, USA) for 15 min. As rhodamine-conjugated (TRITC) secondary antibody, anti-mouse IgG was added (1:120 Dako A/S, Glostrup, Denmark) for 15 min. Finally, slides were incubated with FITC-conjugated anti-human IgA (1:40, Dako A/S) as above. Samples were examined under an Olympus BX60 microscope.

CD25-positive T-cells

CD25+ regulatory T cells were stained from the *lamina propria* of *ex vivo* cultured small-bowel mucosal biopsy sections by an immunofluorescence method. Prior to stainings, the sections were fixed with cold acetone for 10 min. To block unspecific background, the samples were incubated with goat normal serum (Vactor Laboratories Inc., Burlingame, USA) for 20 min, whereafter mouse monoclonal anti-CD25 antibody (1:25, Dako A/S) was added. After one hour, samples were washed and incubated with FITC-conjugated goat anti-mouse IgG (1:1000, Invitrogen) for 30 min. The density of CD25+ T- cells was calculated and presented as number of cells in a total area of 1 mm^2 of mucosal *lamina propria*.

Enterocyte cell height

For morphological studies, the cultured biopsy samples were fixed with cold acetone-alcohol (1:1) for 3 min and stained with standard hematoxylene (Merck), and incubated for 1-2 min. Enterocyte cell height was measured in each biopsy section by analysing at least 30 different epithelial cells from three different villi. The samples were assessed under Olympus BX60 microscope using the analySIS 3.0 program.

4.7 Statistics

Results are given as medians (**I**), means (**II**, **III)** and standard error of means (**II**, **III**). Statistical differences between study groups were evaluated using two-tailed Wilcoxon Signed Rank test (**I**, **II**) or Mann-Whitney U test (**I**, **II**, **III**); p-values lower than 0.05 was considered statistically significant in all studies.

5. RESULTS

5.1 Toxic effects of gliadin and secalin *in vitro* (I,II,III)

5.1.1 Intestinal epithelial cells

The toxic effects of gliadin and secalin were investigated in different kinds of Caco-2 epithelial cell culture experiments. In the first experiment, PT-G and PT-S equally increased epithelial cell layer permeability, measured as recovery of TER between a confluent Caco-2 cell layer. In this assessment, TER was measured immediately and after the cell cultures were supplemented with the study compounds: medium only, PT-BSA, PT-G and PT-S. Recovery of TER was measured once every hour until reaching the baseline level in control cells cultured with medium only. In all cell cultures, TER dropped after culture media exchange, as demonstrated elsewhere (Li et al., 2003). In control cells cultured with medium only or with PT-BSA, TER recovered close to baseline level after 6 hours (Figure 9). In contrast, cells treated with PT-G or PT-S failed to recover completely, indicating increased permeability of the cell layer in the presence of both of these gluten prolamins.

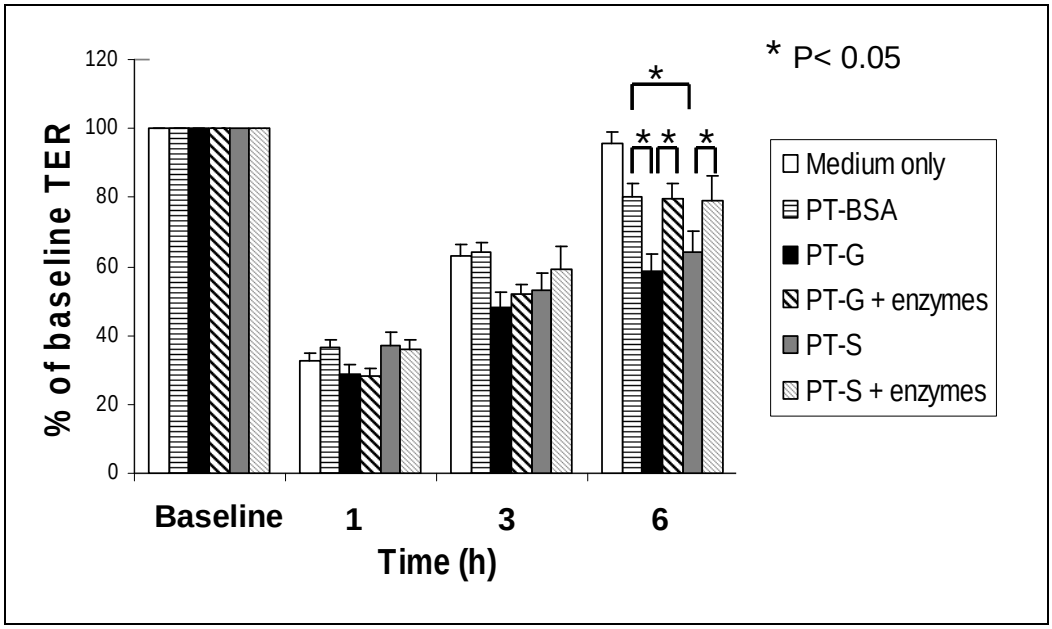

Figure 9. Transepithelial resistance (TER) between Caco-2 cell layers measured before (baseline) and after addition of study compounds: medium only, pepsin-trypsin-digested bovine serum albumin (PT-BSA), pepsin-trypsin-digested gliadin (PT-G), germinating wheat enzyme-pre-treated PT-G (PT-G + enzymes), pepsin-trypsin-digested secalin (PT-S) or germinating barley enzyme-pre-treated PT-S. Data are presented as mean percentages of baseline TER values + standard error of mean. $p<0.05$ was considered significant. * indicates significance of PT-G and PT-S compared to other study compounds.

Secondly, PT-G and PT-S similarly altered actin organisation (Figure 10) as well as the expression of the tight junction-associated proteins occludin and ZO-1 (Figure 10). In these experiments, addition of PT-G or PT-S stimulated actin membrane ruffling at the edges of the cell layers in comparison to control cells, administered to medium only or PT-BSA, where the cell edges remained fairly intact after 24h incubation with the study compounds (Figure 10). In addition, PT-G and PT-S induced straightening and weakening of the expression of occludin and ZO-1 proteins in contrast to the curly appearance in the control cells cultured with medium only or PT-BSA (Figure 10). Of note, it was observed in all experiments that pepsin and trypsin pre-treament alone also had minor effects on Caco-2 cells, since also the negative control, PT-BSA had an influence on these cells.

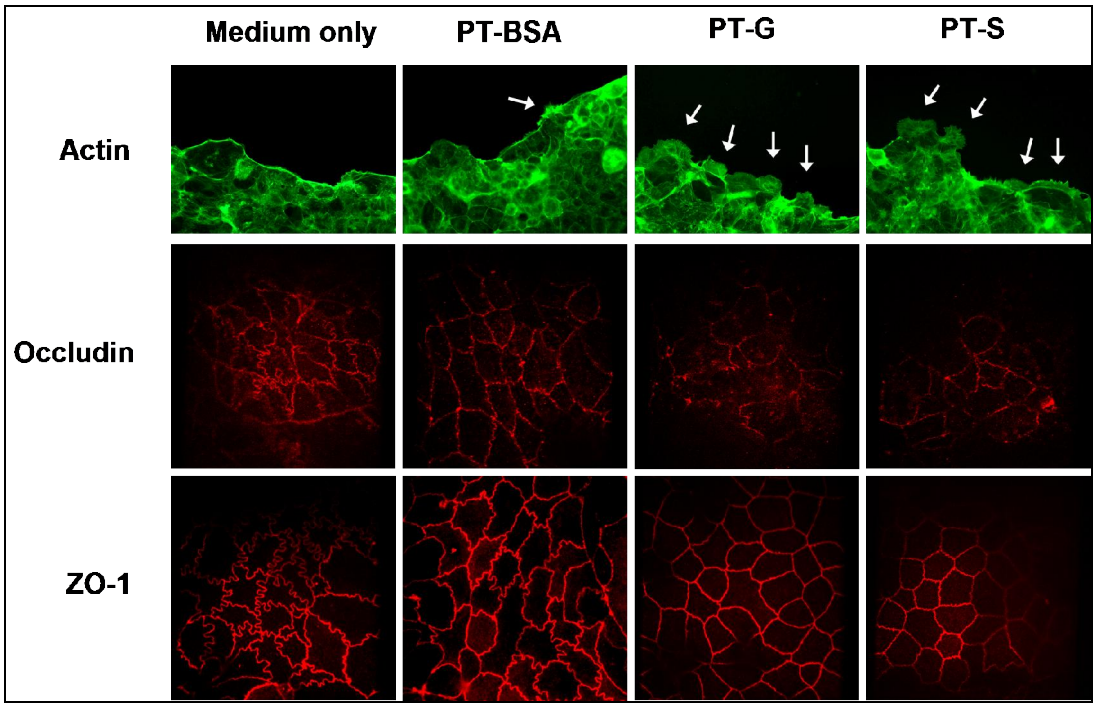

Figure 10. Representative figures of actin cytoskeleton reorganisation and expression of tight junction proteins occludin and ZO-1 in intestinal Caco-2 epithelial cells supplemented with the study compounds: medium only, pepsin-trypsin-digested bovine serum albumin (PT-BSA), pepsin-trypsin-digested gliadin (PT-G) and pepsin-trypsin-digested secalin (PT-S).

Furthermore, the secretion of the innate immunity-related cytokine IL-15 was measured in Caco-2 cell culture supernatants after the cells were treated with medium only, PT-BSA, PT-G or PT-S for distinct time periods (6h, 24h, 48h). The cytokine levels, however, were not measurable by ELISA, since no detectable amount of IL-15 could be seen in any of the cell cultures (**III**).

5.1.2 Small-bowel mucosal biopsies

The small-bowel mucosal IgA deposits, targeted againts TG2, were stained in the biopsies from all participants (**I**) before and after 24h organ culture. Immunofluorescence stainings revealed that all 5 untreated coeliac disease patients had strong IgA deposits below the epithelial basement layer and around mucosal blood vessels already prior to the *in vitro* gliadin challenge. After 24h organ culture, the intensity of IgA deposits did not change from baseline (Figure 11). In the group of treated coeliac disease patients, IgA deposits were found in 11 out of 20 cases, measured from baseline. It was generally seen that the 11 cases with positive IgA deposits had been on a short-term gluten-free diet (GFD 1-4 years) whereas the remaining nine patients on long-term diet (GFD 4-20 years) showed no IgA deposits (Figure 10). The intensity of IgA staining varied among the treated coeliac disease group from weak positive to strong positive. However, there was no difference between biopsies cultured with or without PT-G, indicating that gliadin did not increase the amount of IgA deposits in the small-bowel mucosa within the time-frame of 24h (data not shown). Finally the group of six non-coeliac controls showed no IgA deposits either before or after organ culture (Figure 11).

In order to demonstrate coeliac disease-specific autoantibody production by *ex vivo* PT-G challenge, EmA and TG2-ab were measured in the organ culture supernatants of biopsies from untreated coeliac disease patients, treated coeliac disease patients and non-coeliac controls. During study **I** it was observed that autoantibodies were secreted by all biopsies derived from untreated coeliac disease patients, but by only 55% of treated coeliac disease cases and none of the non-coeliac controls (Table 1 in original paper **I**). It was also seen that autoantibody secretion could be fully stimulated only after a 48h gliadin challenge (Tables 2 and 3 in original paper **I**). When investigating the autoantibody production in greater detail, it was noted that EmA and TG2-ab secretion was related to the duration of patients' gluten-free diet and the presence of small-bowel mucosal IgA deposits (Figure 11, Table 7). Autoantibody production could thus be seen only in those

patients who still had mucosal IgA deposits. Such cases were generally short-term-treated (GFD 1-4 years).

In untreated and short-term-treated cases, EmA and TG2-ab secretion significantly increased after PT-G administration to the culture supernatant (Table 7), although some spontaneous detachment of the autoantibodies was also seen in biopsies cultured with medium only (Table 7). Similarly, PT-G significantly increased the density of *lamina proprial* CD25+ T cells in the cultured small-bowel mucosal biopsies (Table 7) and reduced epithelial cell height (Table 7). These parameters were also dependent on pre-existing IgA deposits and were not to be seen so extensively in long-term-treated coeliac patients or non-coeliac controls.

.

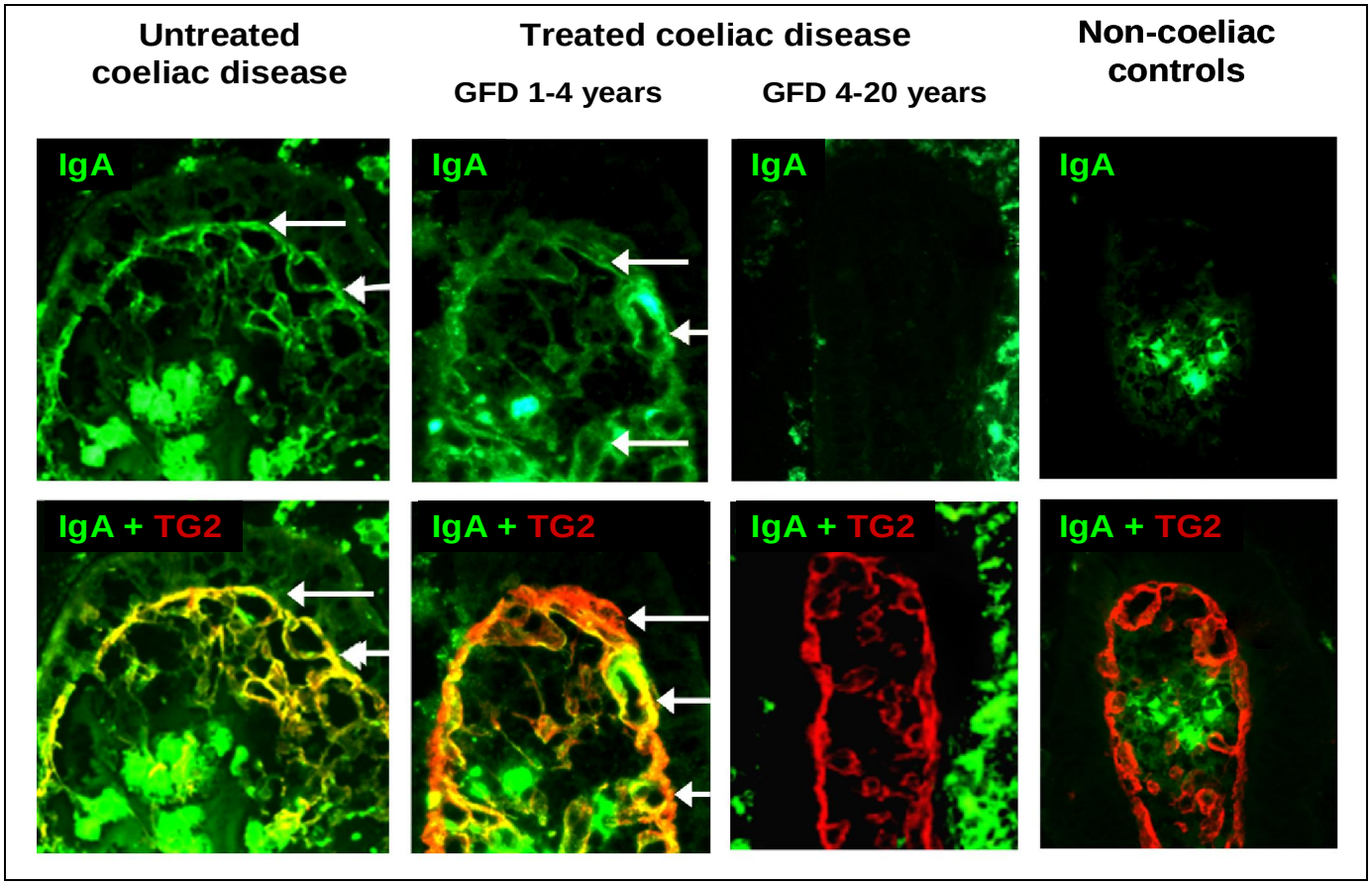

Figure 11. Small-bowel mucosal IgA deposits (green, arrows) targeted against transglutaminase 2 (TG2, red) in biopsy sections of ex vivo cultured biopsies from untreated coeliac disease patients, treated coeliac disease patients and non-coeliac controls. Co-localisation of IgA and TG2 is indicatred in yellow.

Table 7. Summary of results from ex vivo small-bowel mucosal biopsy culture from studies **I** and **II**. The biopsies were obtained either from untreated coeliac disease patients, treated coeliac disease patients with varying duration of gluten-free diet or non-coeliac controls. Median values of endomysial antibodies (EmA) and transglutaminase 2 antibodies (TG2-ab) in organ culture supernatant, CD25 positive T cells in the mucosal lamina propria and enterocyte cell height of cultured biopsies cultured with or without gliadin.

	Untreated coeliac disease		**Treated coeliac disease**				**Non-coeliac controls**	
			GFD 1-4 years		**GFD 4-20 years**			
	Medium only	**PT-G**	**Medium only**	**PT-G**	**Medium only**	**PT-G**	**Medium only**	**PT-G**
IgA deposits	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative
EmA in supernatants (median)	+++ (n=5)	+ + + + (n=5)	+ (n=11)	+++ (n=11) p<0.01	Negative (n=9)	Negative (n=9)	Negative (n=6)	Negative (n=6)
Study I	(**I**)	(**I**)	(**I**)	(**I**)	(**I**)	(**I**)	(**I**)	(**I**)
TG2-ab in supernatants (median, U)	54.1 (n=16)	117.2 (n=16) p<0.01	11.0 (n=11)	24.5 (n=11) p=0.011	2.5 (n=9)	4.4 (n=9)	3.6 (n=9)	0.7 (n=9)
Study I,II	(**I+II**)	(**I+II**)	(**I**)	(**I**)	(**I**)	(**I**)	(**I+II**)	(**I+II**)
CD25+ T cells (median/mm^2)	89 (n=11)	125 (n=11) p=0.021	145 (n=11)	208 (n=11) p=0.008	59 (n=9)	128 (n=9) p=0.058	44 (n=9)	28 (n=9)
Study I,II	(**II**)	(**II**)	(**I**)	(**I**)	(**I**)	(**I**)	(**I+II**)	(**I+II**)
Enterocyte cell height (median, mm)			29,1 (n=7)	23,8 (n=7) p=0.028	26,9 (n=7)	27,5 (n=7)	26,6 (n=6)	27,2 (n=6)
Study I			(**I**)	(**I**)	(**I**)	(**I**)	(**I**)	(**I**)

EmA=endomysial antibodies; GFD=gluten-free diet, PT-G=pepsin-trypsin-digested gliadin; TG2-ab=transglutaminase 2 antibodies

5.2 Detoxification of gliadin and secalin (II,III)

5.2.1 HPLC-MS and SDS-PAGE

In order to detoxify gliadin and secalin peptides, these prolamins were pre-treated with different concentrations of germinating cereal enzymes originating from three cereals: wheat, oats or barley. All three germinating cereal enzymes cleaved full gliadin and secalin into short peptides, seen in HPLC-MS in the disappearance of late-eluting peptide peaks after 20 min, illustrating long multivalent peptides (Figure 12A and B). Of these, the barley enzymes were superior in the cleavage process (Figure 12A and B). In comparing the amounts of different germinating cereal enzymes needed to cleave the full gliadin or secalin by 50% (EC_{50}), germinating barley enzymes were seen to be the most efficient, degrading both prolamins in comparison to germinating wheat and oat enzymes (Table 1 in original paper **III**). Similar results were seen in SDS-PAGE, where hardly any peptides were left after gliadin was pre-treated with germinating wheat enzymes (Figure 1E in original paper **II**) and secalin with germinating barley enzymes (Figure 1C in original paper **III**). Although the real differences between the different enzymes were rather small, we continued using germinating wheat and barley enzymes in further experiments.

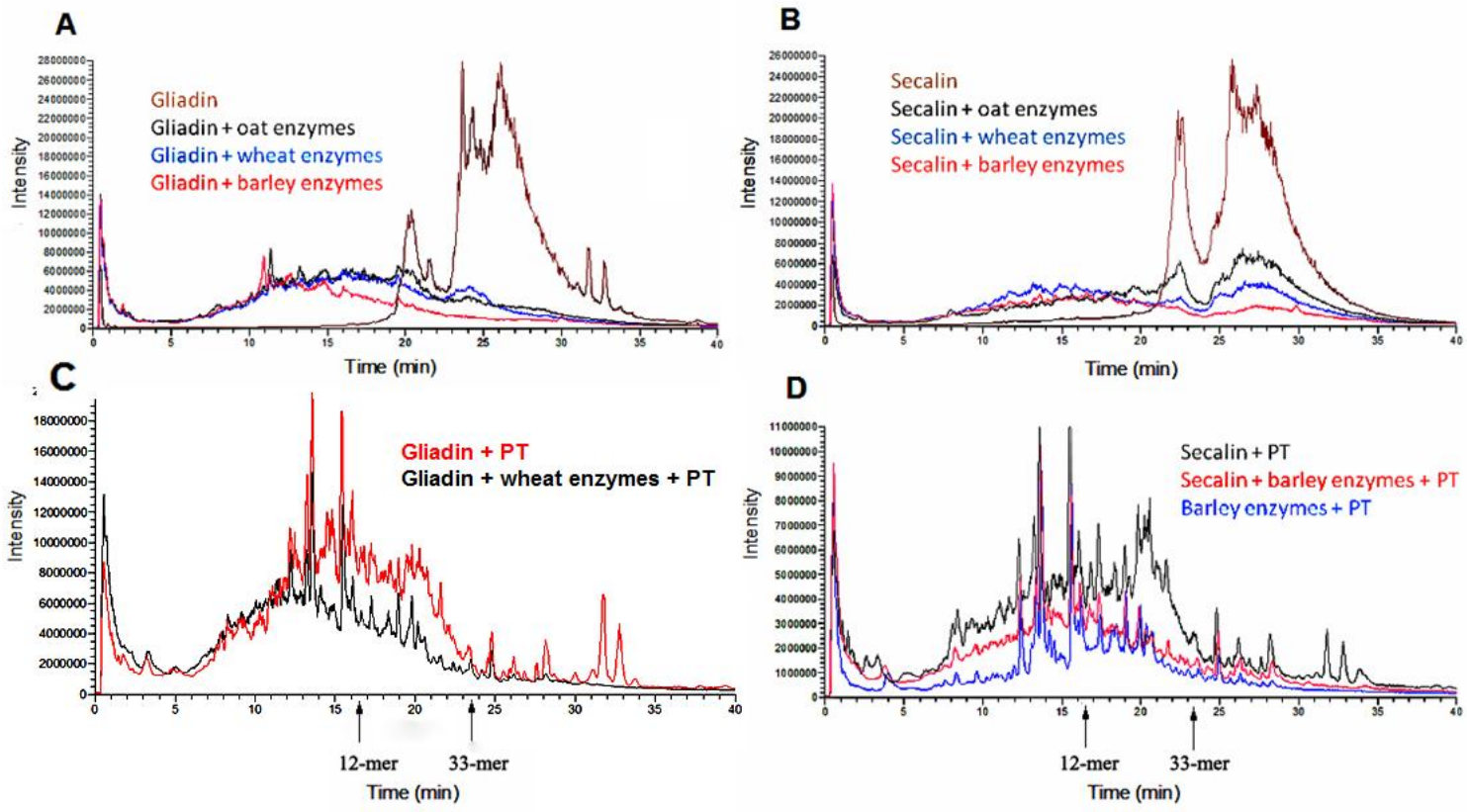

Figure 12. HPLC-MS elution profiles illustrating degradation of gliadin (A,C) and secalin (B,D) pre-treated with germinating enzymes from wheat, oats or barley. Retention times for standard α-gliadin peptides 12-mer and 33-mer are shown by arrows. PT=pepsin-trypsin-digestion.

Digestion of gliadin and secalin were studied in greater detail in HPLC-MS. Pre-treatment of PT-gliadin with germinating wheat enzymes accelerated the degradation of peptides compared to pepsin-trypsin treatment alone (Figure 12C). Similarly, highly efficient degradation of secalin occurred after pre-treatment with germinating barley enzymes (Figure 12D). The majority of long peptides such as coeliac disease-activating α-gliadin peptides 12-mer and 33-mer, eluting after 15 min, had disappeared. Only remaining amounts of cleavage products of pepsin, trypsin and the enzymes themselves were seen in the profiles.

5.2.2 Intestinal epithelial cells

In seeking to establish whether enzymatically pre-treated gliadin and secalin products increase epithelial cell layer permeability in Caco-2 cells, it was observed that this effect was completely abolished. When gliadin was pre-treated with germinating wheat enzymes (**II**) and secalin with germinating barley enzymes (**III**), TER recovered close to baseline level after 6 hours, similarly to negative controls (Figure 9). In this experiment, the germinating cereal enzymes themselves did not affect the TER (data not shown).

In the second cell culture experiment, the magnitude of actin cytoskeleton rearragement was calculated as a percentage of Caco-2 cell cluster edges covered by membrane ruffles. It was seen that pre-treatment with germinating cereal enzymes clearly diminished the effects of PT-G and PT-S (Figure 13). In cells cultured with unprocessed PT-G there was high amount of ruffle formation in comparison to PT-G pre-treated with germinating oat, wheat or barley enzymes, the ruffle formation remained at the level of negative control, PT-BSA (Figure 13). Similar results were obtained when secalin was pre-treated with germinating oat, wheat or barley enzymes. The germinating cereal enzymes alone did not increase the amount of membrane ruffles (Figure 13).

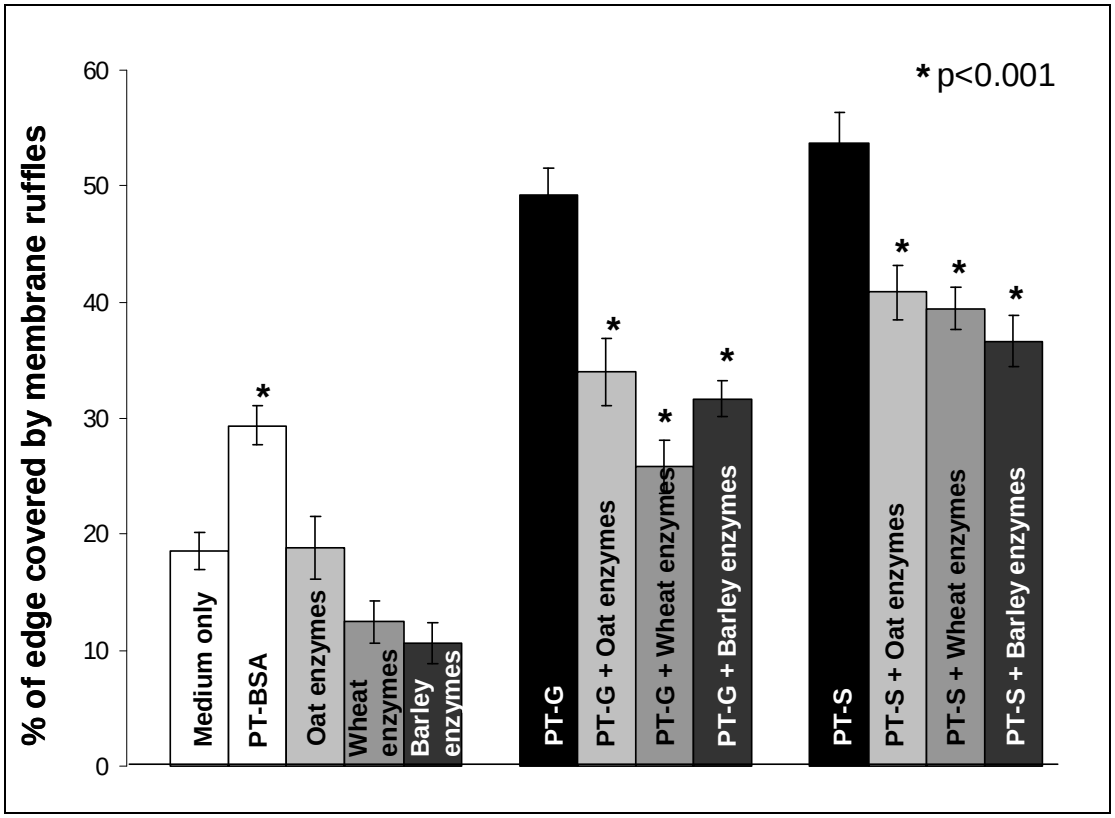

Figure 13. Actin membrane ruffles at the edges of Caco-2 cell clusters measured as percentages of the ruffle length in comparison to the total length of the cell layer. Cells were treated either with medium only, pepsin-trypsin-digested bovine serum albumin (PT-BSA), germinating oat enzymes, germinating wheat enzymes, germinating barley enzymes, pepsin-trypsin-digested gliadin (PT-G), germinating oat enzyme pre-treated PT-G, germinating wheat enzyme-pre-treated PT-G, germinating barley enzyme pre-treated PT-G, pepsin-trypsin-pre-treated secalin (PT-S), germinating oat enzyme-pre-treated PT-S, germinating wheat enzyme pre-treated PT-S or germinating barley enzyme-pre-treated PT-S. Data are given as mean values ± standard error of mean. $p<0.05$ was considered significant. * indicates $p<0.001$ significance compared to PT-G and PT-S.

Finally, pre-treatment of PT-G and PT-S with germinating cereal enzymes was able to normalise the appearance of tight junction-associated proteins occludin and ZO-1 to some extent to the level of negative control PT-BSA (Figure 4 in original paper **II** and Figure 3 in original paper **III**). Again, addition of germinating cereal enzymes alone did not interfere with tight junction formation.

5.2.3 Patient-derived T cell lines and clones

PT-G increased the proliferation of two CD4+ patient-derived TCLs and two TCCs reactive to either native or TG2 deamidated gliadin peptides. After pre-treatment of PT-G with germinating wheat enzymes, the proliferation was reduced in all TCLs and TCCs (Figure 5 in original paper **II**). Addition of negative control, PT-BSA, did not stimulate T cell proliferation in any of the TCLs or TCCs.

5.2.4 Small-bowel mucosal biopsies

Finally, the toxicity of gliadin cleavage products was tested in *ex vivo* cultured small-bowel mucosal biopsies from untreated coeliac disease patients (n=22). In the first assessment, PT-G significantly stimulated TG2-ab production in organ culture supernatans in comparison to biopsies cultured with medium for only 48h. However, when PT-G was pre-treated with germinating wheat enzymes, the autoantibody production remained at the level of the control with medium only (Figure 6A in original paper **II)**. TG2-ab production was scarcely to be seen in biopsies of non-coeliac participants.

Similarly, PT-G significantly increased the number of *lamina proprial* CD25 positive T cells in the small-intestinal mucosal biopsies from untreated coeliac disease patients cultured in the organ culture system for 24h. This harmful effect was diminished when PT-G was pre-treated with germinating wheat enzymes (Figure 6B in original paper **II**). No significant changes were seen from biopsies of non-coeliac participants.

6. DISCUSSION

6.1 Cell and organ culture models for coeliac disease

6.1.1 Small-intestinal epithelial cells

It would be optimal to culture coeliac patient-derived epithelial cells, but it remains complicated to maintain differentiated intestinal epithelial cells in *ex vivo* culture, since the cells remain viable only short-term after detachment due to their rapid apoptosis (Kaeffer, 2002). The intestinal epithelium is actually the most rapidly self-renewing tissue in mammals. In contrast, cancer cell lines can be maintained in culture conditions for weeks, although they do not necessarily originate from patients suffering from the disease of interest, and furher might have accelerated metabolism and other properties which do not fully correspond to normal cell behaviour. Nonetheless, the human intestinal colon carcinoma cell line Caco-2 cells are of small-intestinal origin, and this cell line has been widely used as an epithelial layer model for gluten-induced alteration in the small-intestinal mucosa related to coeliac disease (Table 2). This notwithstanding, it is not known whether these cells actually originate from an individual suffering from coeliac disease, most probably not.

Recently, the innate immunity aspect has become an increasing important issue in the discussion of coeliac disease pathogenesis (Jabri et al., 2000; Maiuri et al., 2000; Raki et al., 2006; Barone et al., 2007). In the present study we demonstrated several early effects of gliadin on intestinal Caco-2 cells for example increased epithelial cell layer permeability and alterations in the expression of F-actin and the tight junction-related proteins occludin and ZO-1 (**II**, **III**). These results are in line with those of previous studies (Clemente et al., 2003; Pizzuti et al., 2004; Sander et al., 2005; Ciccocioppo et al., 2006; Barone et al., 2007). However, it is challenging to point out the major roles of the above mentioned effects on development of the

disorder. First of all, it is not fully known in which order all the above mentioned innate and adaptive immunity reactions occur in coeliac disease. If gliadin has the potency to stimulate very early cascades independently from T cells, as it seems according to the present study, the harmfulness of gluten might be strongly related to excessive leakage of gluten peptides into mucosal *lamina propria* due to increased epithelial layer permeability. The altered appearance of epithelial junctions and actin cytoskeleton support this hypothesis. Further studies are recommented to explore detailed mechanisms behind the changes in the enterocyte level. In addition, it remains to be solved whether the above mentioned early events stimulated by gliadin also occur *in vivo* in coeliac disease. In the current series, it would also have been worth studying more relevant innate immunity components, but for example measurement of IL-15 from Caco-2 cell culture supernatants turned out to be impracticable (**III**). It has in fact been reported that IL-15 is poorly secreted from cultured epithelial cells (Maiuri et al., 2000; Mention et al., 2003).

6.1.2 Small-bowel mucosal biopsies

The epithelial cell cultures represent only a minor part of the whole intestinal system. To mimic the *in vivo* conditions of the small intestine more closely, it is preferable to isolate the mucosal biopsy compartment directly from individuals having the disease of interest. The advantage of this kind of *ex vivo* cultured mucosal biopsies is that the biopsies include a diversity of mucosal cell types from stem cells to highly differentiated epithelial cells and immune cells, which can furthermore communicate with each other. The major limitations for the organ culture system are, however, lack of circulation and nervous system as well as contact with other lymphatic tissues. In spite of these drawbacks, the organ culture system is still probably the most relevant model to mimic *in vivo* conditions in the small-bowel mucosa in coeliac disease.

The human organ culture method has been widely utilised in the field of coeliac disease research since it was first presented in 1969 (Browning and Trier, 1969). It has been illustrated that gluten induces a number of harmfull effects in mucosal

biopsies from both untreated and treated coeliac disease patients, but not in non-coeliac controls (Table 3). Unfortunately, there has been controversy as to whether these effects are actually dependent on gliadin administrations and whether these phenomena can be demonstrated in all coeliac disease patients. It has previously been reported that for example production of coeliac disease-specific antibodies can be demonstrated in only some of the treated coeliac disease patients participating in a study (Vogelsang et al., 1999; Biagi et al., 2000; Carroccio et al., 2002), this rendering the functionality of the method somewhat questionable.

In our hands, EmA and TG2-ab were secreted to culture supernatants in 11 out of 20 treated coeliac disease cases (**I**) and in all untreated coeliac disease cases (**I**, **II**). In closer analysis of the cases, it was clearly seen that the majority of the toxic effects of gliadin could be demonstrated only in those coeliac disease cases who were short-term treated and who still had small-bowel mucosal IgA deposits in the cultured biopsy sections. This suggests that the presence of at least some components of the adaptive immune system might be needed to activate downstream effects in the cultured mucosal biopsies. Moreover, it is not known whether contact with the circulation and other lymphatic tissues are essential for adaptive immunity mechanisms to operate as they do in *in vivo* conditions. Another important fact is that the 24 or 48 hours *ex vivo* methodology may not allow sufficient time for all significant reactions to take place. Even though PT-gliadin significantly stimulated immune reactions in cultured biopsy samples from coeliac disease patients, there was for example spontaneous detachment of the autoantibodies from biopsies cultured without any supplementation (**I, II**). A longer time than 48h would thus be preferable to bring out even greater differences between the study compounds. However, in culturing the biopsies in the current system for more than 48h, the morphology of the samples deteriorates markedly due to necrosis and apoptosis of the tissues, seen also in the preliminary organ culture studies (Jos et al., 1975).

While keeping these limitations in mind, the present studies showed the organ culture system to be a relevant tool for the study of coeliac disease *ex vivo*. However, it is preferable to combine different primary cell cultures and more

complex three-dimensional tissue and organ culture methods if the objectives are to demonstrate the overall harmful effects of gluten on the human small-intestinal mucosa in the context of coeliac disease.

Another important issue arising after the present study was the role of small-bowel mucosal IgA deposits in coeliac disease pathogenesis. Although the pathogenetic aspect was beyond the scobe of the current study, the role of the IgA deposits is clearly a matter for investigation in the future. It is for example interesting why the IgA deposits targeted against TG2 are located around the blood vessels and right below the epithelial layer. They might have a protective role by blocking of gluten intake into *lamina propria* or they may disturb normal cell behaviour such as epithelial and endothelial cell layer permeability, as shown in previous studies (Halttunen and Mäki, 1999; Zanoni et al., 2006; Myrsky et al., 2008; Myrsky et al., 2009). It may also be important to investigate if there exist novel functions for TG2 in the small-intestinal mucosa and why IgA is targeted towards this specific enzyme. It has previously been noted *in vivo* that these IgA deposits already appear in a very early phase of the disease procress (Korponay-Szabo et al., 2004; Kaukinen et al., 2005; Salmi et al., 2006a) but disappear slowly on a the gluten-free diet (Salmi et al., 2006b). The same phenomenon was also seen in the current series: in some treated coeliac disease cases, IgA deposits were found even after four years on a gluten-free diet (**I**). However, the intensity of IgA deposits below the mucosal epithelial basement membrane or around vessels did not increase after *ex vivo* gliadin challenge.

6.2 Comparing the toxicity of wheat gliadin and rye secalin *in vitro*

The current knowledge of harmfulness of rye secalin in coeliac disease is based on a few *in vivo* and *in vitro* studies (Dicke et al., 1953; Anand et al., 1978; Kieffer et al., 1982; Bracken et al., 2006; Kilmartin et al., 2006). The early patient-based studies are, however, limited by the very small number of patients involved and the methods used are not necessarily as specific for coeliac disease diagnosis as those used today. More recently, secalin was shown to activate adaptive immunity

components such as IFN-γ and IL-2 expression in *ex vivo* cultured small-bowel mucosal biopsies from coeliac disease patients (Bracken et al., 2006) and coeliac patient derived T cell lines (Kilmartin et al., 2006). Moreover, in a study by Kilmartin and associates (2006), different cereal prolamines (gliadin, avenin, secalin and hordein) stimulated T cell proliferation. Interestingly, however, there were also minor differences between the four prolamines. In some cases, proliferation of TCL was only slighlty increased in cells treated with avenin, secalin and hordein compared to the more extensive activation when treated with gliadin. It may thus be assumed that the overall toxicity of secalin and hordein *in vivo* might differ from that of gliadin. The most recent study concerning the toxicity of *triticeae* cereals included a systematic epitope mapping of wheat, rye and barley prolamins using coeliac-patient derived peripheral blood monocytes to find all harmful sequences causing coeliac disease in adults (Tye-Din et al., 2010). Nevertheless, none of the above-mentioned studies has focused on the ability of secalin to stimulate innate immunity reactions in the small-bowel mucosa of coeliac disease patients. In the present study (**III**), secalin was shown also to elicite early effects on intestinal cells: it increased epithelial cell layer permeability, actin membrane ruffling and altered tight junctional protein occludin and ZO-1 expressions in intestinal Caco-2 cells. More interestingly, all the toxic effects were as intensive as those stimulated by wheat gliadin, indicating that secalin also exerts a strong influence on innate immunity reactions in intestinal epithelial cells. In addition, these results further support those of previous studies where it has been staited that rye should be abandoned from the gluten-free diet.

6.3 Novel treatment for coeliac disease

A great many different alternative treatment strategies for coeliac disease have been introduced during recent years (Table 1). Many of them concentrate on gluten modification before the harmful gluten peptides enter the small-bowel mucosa. Genetic engineering of cereals has also been planned to improve the quality of gluten-free products (Vader et al., 2003; Spaenij-Dekking et al., 2005; Pizzuti et al., 2006; Carroccio et al., 2010). However, in practice such manipulation has been

particularly challenging since gluten proteins contain a diversity of immunotoxic sequences of which not all the toxic peptides are even known (Vader et al., 2003; Shan et al., 2005; Tye-Din et al., 2010). Subsequently endogenous enzymes have been introduced to accelerate gluten degradation (Shan et al., 2002). Additional enzymes together with human digestive proteases could cleave gluten peptides totally during the ingestion of food. Several external enzymes of different origins have been proposed to detoxify gluten epitopes (Table 1).

In order for an oral enzyme therapy to be effective in detoxifying gluten, it would have to fulfil the following criteria: 1) the enzyme supplementation should almost completely detoxify gluten before it enters into the small-intestinal mucosa, 2) the enzyme preparation should be effective under both gastric and duodenal conditions and resistant to gastrointestinal digestive enzymes and bile acids, 3) the concentration of the enzyme preparation and the exposure time should be reasonable, and 4) the manufacturing process should be feasible and economical (Sollid and Khosla, 2005). Furthermore, problems arising from the divergent gastric emptying times in individuals and the distinct susceptibility of coeliac patients to tiny amounts of residual gluten might also arise in clinical practice (Catassi et al., 2007).

6.3.1 Detoxification of gluten by germinating cereal enzymes

The aim of the current work was to show proof of concept for enzymatic detoxification of gluten prolamins (**II**, **III**). An array of germinating cereal enzymes from oats, wheat or barley were selected to degrade gliadin and secalin. The major advantage of these prolamins is that they are evolutionarily selected to effect total cleavage of the cereal storage proteins in germinating kernels. As shown in HPLC-MS and SDS-PAGE, these enzymes were particularly efficient in hydrolysing gliadin and secalin also in laboratory conditions, since only short peptides, probably no longer than 10 amino acids, were left after 24h pre-treatment with the above-mentioned germinating cereal enzymes (**II**, **III**). It has been proposed that a large

fraction of the gliadin peptides recognised by coeliac disease-specific T-cells are between 21 and 30 residues in length, whereas short peptides (1-10 residues) are nonstimulating (Marti et al., 2005). It may thus be assumed that the bulk of the overall amount of coeliac disease-inducing gluten peptides were degraded after enzymatic pre-treatment.

Although germinating barley enzymes were superior in the cleavage of both gliadin and secalin (**III**), there were only minor differences between the three enzyme mixtures (oats, wheat and barley). However, since the aim of the current study was solely to demonstrate proof-of-concept for the notion of using the germinating cereal enzymes, the process of degradation of gliadin and secalin was not extensively optimised. Instead we chose the most efficient enzymes for further experiments. It is assumed that all three enzyme mixtures might be utilised in gluten detoxification. It might also be useful to combine these enzymes or enrich the most important single proteases. In the course of the study it was realised that it would have been also worth investigating the efficacy of germinating rye enzymes in hydrolysing gluten prolamins, especially secalin. In another study, the activity of germinating rye enzymes turned out to be higher than other germinating cereal enzymes (Hartmann et al., 2006; Loponen et al., 2009). On the whole, it seems that co-administration of several enzymes is superior in the full breakdown of gluten peptides instead of using only one specific enzyme (Siegel et al., 2006; Gass et al., 2007; Ehren et al., 2009).

After germinating cereal enzyme pre-treatment, the toxicity of PT-gliadin and PT-secalin was markedly diminished *in vitro*, as measured in several Caco-2 epithelial cell culture experiments, a patient-derived T cell proliferation assay as well as *ex vivo* cultured small-bowel mucosal biopsies of coeliac disease patients. In addition, the differences in the efficacy of germinating oats, wheat and barley enzymes in detoxifying gliadin and secalin was also analysed in the Caco-2 cell culture assay, where actin membrane ruffling was detected. In the current experiment, all three enzyme pre-treatments resulted in significantly diminished membrane ruffling (Figure 13), indicating in practice only minor differences between the enzyme preparations. Even though germinating cereal enzymes efficiently abolished activation of innate immunity reactions, some adaptive

immunity reactions continued to occur after enzyme pre-treatment of gliadin (**II**). These were for example a minor increase in T cell proliferation and activation of CD25+ T cells in *ex vivo* cultured small-bowel mucosal biopsies from coeliac disease patients. It would thus appear that not all stimulatory gliadin epitopes were completely eliminated after pre-treatment with germinating wheat enzymes (**II**). Subsequently it emerged that a small amount of highly immunogenic 33-mer, and possible some other peptides still remained in the gliadin samples pre-treated with germinating wheat enzymes.

Gluten detoxification by germinating cereal enzymes offers a promising alternative means of treating coeliac disease in the future. The approach might be especially helpful during occasional or accidental ingestion of small amounts of gluten for example during travelling and social events (Matysiak-Budnik et al., 2005). In addition, this mechanism could be utilised in food processing in order to eliminate gluten toxicity and to develop high-quality coeliac-safe food products. Further studies are needed to determine whether these enzymes can be used as a full alternative treatment, replacing the gluten-free diet challenging for gluten-sensitive people.

7. CONCLUSIONS AND FUTURE CHALLENGES

At present, *in vitro* studies in the field of coeliac disease rely on different intestinal epithelial cancer cell lines, patient-derived T cell lines and clones and *ex vivo* cultured small-bowel mucosal biopsies from coeliac disease patients. A number of attemps have also been made to develop a functional disease-specific animal model (Marietta et al., 2004; Di Niro et al., 2008; Verdu et al., 2008; de Kauwe et al., 2009; Freitag et al., 2009). Nonetheless, all these models have limitations due to which they do not fully correlate to the circumstances in the human small intestine *in vivo*. For example primary cell cultures lack connections to other cell types and thus signalling to other cell layers. In general cancer cells and animal cells differ to some extent from normal human cell behavior. In addition, cell and organ culture models lack circulation, nervous system and conncection to lymphatic organs which might mean absence of some crucial components in the development of a particular disease *in vivo*.

Therefore, in the future it would thus be extremely important to promote disease-specific *in vitro* models and develop novel culture methods. For example, it would be ideal to culture coeliac disease-derived epithelial cells, instead of those originating from cancer cells. Several studies have already introduced a mechanism for the isolation and short-term culturing of small-bowel mucosal epithelium from patients with coeliac disease (Mention et al., 2003; Di Sabatino et al., 2006). However, since coeliac disease develops slowly and gradually, it takes probably far more time than 24 or 48 hours' for all cellular reactions to occur, long-lived cultures would be preferred to study the appearance of diverse mucosal changes after gluten administration. However, generally fully differentiated intestinal epithelial cell populations grow poorly in current cell culture conditions (Kaeffer, 2002). But interestingly, very recent studies have introduced a long-term renewal of gastric

mucosa by culturing of mice small-intestinal Lrg5-positive stem cells which are able to differentiate and form villous-like small-intestinal epithelial cells (Sato et al., 2009; Barker et al., 2010). These kinds of cells might be included in the coeliac disease research especially if similar type of cells could be isolated from the human gut. All in all, researchers must overcome the challenges attending culture conditions with the help of tissue engineering and other novel techniques.

These deficts notwithstanding, the above mentioned *in vitro* systems remain highly valuable during the development and testing of alternative treatment strategies for coeliac disease. Before clinical trials, all potential drug candidates are in first place tested by using different cell and organ culture disease models. Currently several novel medical treatment strategies for coeliac disease are already in Phase I or II clinical trials (Pyle et al., 2005; Paterson et al., 2007; Daveson, 2009; Tye-Din et al., 2009). However, it takes years for a drug candidate to come onto the markets. Most of preliminary concepts drop out during different development stages. The future for the strategy envisaged in the current study concerning germinating cereal enzymes as a novel treatment for coeliac disease, depends on furher optimisation and testing in cell culture and animal models. It may be useful to enrich certain specific enzymes or combine them with others introduced in previous studies. Eventually this method would seem to have every prospect of moving on to preliminary clinical trials. One of the germinating barley enzymes, EP-B2, discovered by Bethune and co-authors (2006), is already in Phase II clinical trials (Tye-Din et al., 2009). It will be seen in the future whether different enzymes used as therapy for coeliac disease can really detoxify all gluten peptides also in *in vivo* conditions and how these enzymes maintain their activity in the diverse pH ranges found in the human digestive tract.

Another important aspect is that all previous studies concerning gluten detoxification have focused on modulation of wheat gliadin. The present study thus brings novel knowledge regarding the degradation of other cereals. Interestingly, the preliminary results of the present series indicated that secalin is about three times more efficiently cleaved than gliadin when using similar constructions of germinating cereal enzyme preparations (Table 1 in original paper **III**). As there

might be significant differences between the degradation efficacies of various cereal prolamins, degradation of other cereals instead of gliadin might be more easily effected.

Gluten-free food products are often of poor quality, have a short self-life and lack taste, so that improvement of baking quality is warranted. The development of grains possessing no immunotoxic sequences but reasonable baking quality is however challenging. It has been proposed that cereal varieties such as *Triticum monococcum*, the oldest and most primitive cultivated wheat, as well as *Triticum durum*, could be administered in the gluten-free diet (Pizzuti et al., 2006; Silano et al., 2007; Silano et al., 2008). *Triticum monococcum* lacks for example the immunogenic 33-mer and could thus be more easily tolerated by patients suffering from coeliac disease (Frisoni et al., 1995; Silano et al., 2007). In addition, sourdough fermentation, during which cereal protein degradation occurs, might provide a novel technique for the improvement of gluten-free products. Use of sourdoughs in baking might provide new whole-grain ingredients with low prolamin content for coeliac disease patients and improve the quality and taste of gluten-free foods (Loponen et al., 2007). Rye, for example, could enhance the flavour, texture and nutritional quality of products by its increased amount of fibre. Preliminary baking trials using germinating rye sourdoughs have already shown better taste and volume in oat bread (Loponen et al., 2009), suitable for coeliac disease patients. Similar studies have been conducted using different lines of sourdough *Lactobacilli* (Di Cagno et al., 2004; De Angelis et al., 2006; Rizzello et al., 2007; Di Cagno et al., 2008).

One major problem for gluten-free food processing is the question of the amount of gluten tolerated by coeliac disease patients. It has been estimated that a daily intake of over 200-500mg of gluten induces small-intestinal mucosal damage and inflammation (Catassi et al., 2007). According to European Union standards for gluten-free foods, the maximum level in a product to be labelled gluten-free is 20ppm (mg/kg) and for a low-gluten-containing product 200ppm. It remains to be seen whether novel coeliac-safe foods rich in enzymatically pre-treated gluten can be developed within these limitations.

ACKNOWLEDGEMENTS

This study was carried out at the Medical School, University of Tampere and the Department of Gastroenterology and Alimentary Track Surgery, and the Department of Paediatrics, Tampere University Hospital.

First of all I want to express my deepest gratitude to Professor Markku Mäki, M.D., not only for opportunity to work in the Paediatric Research Centre but offering a unique chance for a young student to jump immediately deeply into scientific world. During these years I have experienced much more than I could ever imagine.

I am particularly grateful to my supervisors Docent Katri Kaukinen, M.D. and Docent Katri Lindfors, PhD, for their amazing optimistic attitude towards all the phases of this study. Especially I thank the patience with me when I needed to have my own time. I can certainly say that without your understanding and supportive role this work would never have completed.

The external reviewers of this thesis, Docent Aki Manninen and Frits Koning, M.D. are sincerely acknowledged for their valuable comments which helped me to improve this thesis. I also want to thank Professor Ilkka Pörsti, M.D. for participating in my thesis committee at the beginning of this project.

I am highly grateful to all my co-authors, Ilma Korponay-Szabo, M.D. Olli Lohi, M.D., Docent Päivi Saavalainen, Docent Jukka Partanen, Katri Haimila, PhD,. Kaija Laurila, M.Sc., Herbert Wieser, PhD, Seppo Auriola, PhD, Timo Mauriala, PhD, Anu Kaukovirta-Norja, PhD, Anna Jantunen, M.Sc., Anne Hautala, M.Sc., Shuo-Wang Qiao, PhD, Ludvig Sollid, M.D., Arturo Garcia-Horsman, PhD and Professor Pekka Männistö. My special thanks go to Jarkko Venäläinen, PhD, for interesting collaboration during these years and for several discussions we have had. I also thank you for revision of manuscripts and technical support with the methods included in the present study.

I also want to express my appreciation to Professor Hannu Salovaara, Tuula Sontag-Strohm, PhD and Jussi Loponen, PhD, for guidancing me into field of cereal technology. I have had several pleasant discussions with you.

I am thankful to Mr. Robert MacGilleon, M.A. for remarkably careful revision of the language of all original papers and this thesis.

I also want to express my sincere thanks to all patients who participated in this study during these years.

I am warmly thankful for all members of the Coeliac Disease Study Group. No words are enough to thank Mr. Jorma Kulmala, Kaija Kaskela, M.Sc., Mrs. Anne Heimonen, Mrs. Mervi Himanka and Mrs. Soili Peltomäki for their enormous kindness and help with all I have ever needed. I thank especially my past colleagues Heidi Siekkinen, M.Sc., Essi Myrsky, PhD and Mari Syrjänen, M.Sc. for the most pleasant time in the lab and free time and for friendship we still have. Also my fellow students Kati Lähteelä, M.Sc. and Arto Lähteelä, M.Sc. have been very important to me during these years, we have grown up together.

I am proud of my parents who have encouraged me to challenge myself and not to give up, even if it has sometimes been even burdensome. I also want to thank Mr. Erkki Kallio for introducing me to Professor Markku Mäki in 2004 when this story began. Finally, I thank my siblings, Pekka, Annaleena and Taruliisa, my soulmate Ansku and other friends outside this work. You really have made these years an adventure!

The present research was financially supported by the Competitive Research Funding of the Pirkanmaa Hospital District, the Yrjö Jahnsson Foundation, the Finnish Medical Foundation, the Paediatric Research Foundation, the Finnish Cultural Foundation, the Sigrid Juselius Foundation, the Research Fund of the Finnish Celiac Society, the Academy of Finland Research Council for Health, the European Comission (contact number MRTN-CT-2006-036032) and Tampere Graduate School in Biomedicine and Biotechnology (TGSBB).

Tampere December 2010

Satumarja Stenman

REFERENCES

Aine, L., Mäki, M., Collin, P., and Keyrilainen, O. (1990). Dental enamel defects in celiac disease. J Oral Pathol Med. 19:241-5.

Amundsen, S.S., Monsuur, A.J., Wapenaar, M.C., Lie, B.A., Ek, J., Gudjonsdottir, A.H., Ascher, H., Wijmenga, C., and Sollid, L.M. (2006). Association analysis of MYO9B gene polymorphisms with celiac disease in a Swedish/Norwegian cohort. Hum Immunol. 67:341-5.

Anand, B.S., Piris, J., and Truelove, S.C. (1978). The role of various cereals in coeliac disease. Q J Med. 47:101-110.

Anderson, R.P., Degano, P., Godkin, A.J., Jewell, D.P., and Hill, A.V. (2000). In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. Nat Med. 6:337-42.

Anderson, R.P., van Heel, D.A., Tye-Din, J.A., Barnardo, M., Salio, M., Jewell, D.P., and Hill, A.V. (2005). T cells in peripheral blood after gluten challenge in coeliac disease. Gut. 54:1217-23.

Anderson, R.P. (2008). Development of a vaccine for celiac disease. *In* Frontiers in Celiac Disease. Vol. 12. A. Fasano, R. Troncone, and D. Branski, editors. Karger, Pediatr Adolesc Med, Basel. 172-180.

Arentz-Hansen, H., Korner, R., Molberg, O., Quarsten, H., Vader, W., Kooy, Y.M., Lundin, K.E., Koning, F., Roepstorff, P., Sollid, L.M., and McAdam, S.N. (2000). The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. J Exp Med. 191:603-12.

Arentz-Hansen, H., McAdam, S.N., Molberg, O., Fleckenstein, B., Lundin, K.E., Jorgensen, T.J., Jung, G., Roepstorff, P., and Sollid, L.M. (2002). Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. Gastroenterology. 123:803-9.

Auricchio, S., De Ritis, G., De Vincenzi, M., Mancini, E., Minetti, M., Sapora, O., and Silano, V. (1984). Agglutinating activity of gliadin-derived peptides from bread wheat: implications for coeliac disease pathogenesis. Biochem Biophys Res Commun. 121:428-33.

Baker, P.G., and Read, A.E. (1976). Oats and barley toxicity in coeliac patients. Postgrad Med J. 52:264-8.

Barker, N., Huch, M., Kujala, P., van de Watering, M., Snippert, HJ., van Es, JH., Sati, T., Stange, DE., Begthel, H., van den Born, M., Danenberg, E., van den Brink, S., Korving, J., Abo, A., Peters, PJ., Wright, N., Poulsorn, R. and Clevers H. (2010). Lgr5(+ve) stem cells drive self-renewal in the atomach and build long-lived gastric units in vitro. Cell Stem Cell. 8;6(1):25-36

Barone, M.V., Gimigliano, A., Castoria, G., Paolella, G., Maurano, F., Paparo, F., Maglio, M., Mineo, A., Miele, E., Nanayakkara, M., Troncone, R., and Auricchio, S. (2007). Growth factor-like activity of gliadin, an alimentary protein: implications for coeliac disease. Gut. 56:480-8.

Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J.H., Lanier, L.L., and Spies, T. (1999). Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science. 285:727-9.

Beissbarth, T., Tye-Din, J.A., Smyth, G.K., Speed, T.P., and Anderson, R.P. (2005). A systematic approach for comprehensive T-cell epitope discovery using peptide libraries. Bioinformatics. 21 Suppl 1:i29-37.

Benahmed, M., Meresse, B., Arnulf, B., Barbe, U., Mention, J.J., Verkarre, V., Allez, M., Cellier, C., Hermine, O., and Cerf-Bensussan, N. (2007). Inhibition of TGF-beta signaling by IL-15: a new role for IL-15 in the loss of immune homeostasis in celiac disease. Gastroenterology. 132:994-1008.

Bethune, M.T., Strop, P., Tang, Y., Sollid, L.M., and Khosla, C. (2006). Heterologous expression, purification, refolding, and structural-functional characterization of EP-B2, a self-activating barley cysteine endoprotease. Chem Biol. 13:637-47.

Bethune, M.T., Ribka, E., Khosla, C., and Sestak, K. (2008). Transepithelial transport and enzymatic detoxification of gluten in gluten-sensitive rhesus macaques. PLoS One. 3:e1857.

Bhagat, G., Naiyer, A.J., Shah, J.G., Harper, J., Jabri, B., Wang, T.C., Green, P.H., and Manavalan, J.S. (2008). Small intestinal CD8+TCRgammadelta+NKG2A+ intraepithelial lymphocytes have attributes of regulatory cells in patients with celiac disease. J Clin Invest. 118:281-93.

Biagi, F., Ellis, H.J., Parnell, N.D., Shidrawi, R.G., Thomas, P.D., O'Reilly, N., Corazza, G.R., and Ciclitira, P.J. (1999). A non-toxic analogue of a coeliac-activating gliadin peptide: a basis for immunomodulation? Aliment Pharmacol Ther. 13:945-50.

Biagi, F., Parnell, N.D., Ellis, H.J., and Ciclitira, P.J. (2000). Endomysial antibody production is not related to histological damage after in vitro gluten challenge. Eur J Gastroenterol Hepatol. 12:57-60.

Bjarnason, I., Peters, T.J., and Veall, N. (1983). A persistent defect in intestinal permeability in coeliac disease demonstrated by a 51Cr-labelled EDTA absorption test. Lancet. 1:323-5.

Bolte, G., Wolburg, H., Beuermann, K., Stocker, S., and Stern, M. (1998). Specific interaction of food proteins with apical membranes of the human intestinal cell lines Caco-2 and T84. Clin Chim Acta. 270:151-67.

Bonamico, M., Sabbatella, L., Di Tola, M., Vetrano, S., Ferri, M., Nenna, R., Mariani, P., and Picarelli, A. (2005). Antiendomysial antibody detection in biopsy culture allows avoidance of gluten challenge in celiac children. J Pediatr Gastroenterol Nutr. 40:165-9; discussion 122-4.

Boscolo, S., Lorenzon, A., Sblattero, D., Florian, F., Stebel, M., Marzari, R., Tarcisio, N., Aesclimann, D., Ventura, A., Hadjivassiliou, M. and Tongiorgi, E. (2010). Anti transglutaminase antibodies cause ataxia in mice. PLoS ONE. 5(3):e9698.

Bottari, A., Capocchi, A., Fontanini, D., and Gallechi, L. (1996). Major proteinase hydrolysing gliadin during wheat germination. Phytochemistry. 43:39-44.

Bracken, S.C., Kilmartin, C., Wieser, H., Jackson, J., and Feighery, C. (2006). Barley and rye prolamins induce an mRNA interferon-gamma response in coeliac mucosa. Aliment Pharmacol Ther. 23:1307-14.

Brijs, K., Trogh, I., Jones, B., and Delcour, J.A. (2002). Proteolytic enzymes in germinating rye grains. Cereal Chem. 79:423-428.

Bronstein, H.D., Haeffner, L.J., and Kowlessar, O.D. (1966). Enzymatic digestion of gliadin: the effect of the resultant peptides in adult celiac disease. Clin Chim Acta. 14:141-55.

Browning, T.H., and Trier, J.S. (1969). Organ culture of mucosal biopsies of human small intestine. J Clin Invest. 48:1423-32.

Bruce, S.E., Bjarnason, I., and Peters, T.J. (1985). Human jejunal transglutaminase: demonstration of activity, enzyme kinetics and substrate specificity with special relation to gliadin and coeliac disease. Clin Sci (Lond). 68:573-9.

Carroccio, A., Iacono, G., D'Amico, D., Cavataio, F., Teresi, S., Caruso, C., Di, P.L., Colombo, A., D'Arpa, F., Florena, A., Notarbartolo, A., and Montalto, G. (2002). Production of anti-endomysial antibodies in cultured duodenal mucosa: usefulness in coeliac disease diagnosis. Scand J Gastroenterol. 37:32-8.

Carroccio, A., Di Prima, L., Pirrone, G., Scalici, C., Florena, A.M., Gasparin, M., Tolazzi, G., Gucciardi, A., Sciume, C., and Iacono, G. (2006). Anti-transglutaminase antibody assay of the culture medium of intestinal biopsy specimens can improve the accuracy of celiac disease diagnosis. Clin Chem. 52:1175-80.

Carroccio, A., Di Prima, L., Noto, D., Fayer, F., Ambrosiano, G., Villanacci, V., Lammers, K.M., Lafiandra, D., De Ambrogio, E., Di Fede, G., Iacono, G., and Pogna, N. (2010). Searching for wheat plants with low toxicity in celiac disease: Between direct toxicity and immunologic activation. Dig Liver Dis. doi:10.1016/j.dld.2010.05.005.

Carswell, F., and Ferguson, A. (1972). Food antibodies in serum--a screening test for coeliac disease. Arch Dis Child. 47:594-6.

Catassi, C., Fabiani, E., Iacono, G., D'Agate, C., Francavilla, R., Biagi, F., Volta, U., Accomando, S., Picarelli, A., De Vitis, I., Pianelli, G., Gesuita, R., Carle, F., Mandolesi, A., Bearzi, I., and Fasano, A. (2007). A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. Am J Clin Nutr. 85:160-6.

Choi, K., Siegel, M., Piper, J.L., Yuan, L., Cho, E., Strnad, P., Omary, B., Rich, K.M., and Khosla, C. (2005). Chemistry and biology of dihydroisoxazole derivatives: selective inhibitors of human transglutaminase 2. Chem Biol. 12:469-75.

Chorzelski, T.P., Sulej, J., Tchorzewska, H., Jablonska, S., Beutner, E.H., and Kumar, V. (1983). IgA class endomysium antibodies in dermatitis herpetiformis and coeliac disease. Ann N Y Acad Sci. 420:325-34.

Ciccocioppo, R., Finamore, A., Ara, C., Di Sabatino, A., Mengheri, E., and Corazza, G.R. (2006). Altered expression, localization, and phosphorylation of epithelial junctional proteins in celiac disease. Am J Clin Pathol. 125:502-11.

Ciclitira, P.J., Evans, D.J., Fagg, N.L., Lennox, E.S., and Dowling, R.H. (1984). Clinical testing of gliadin fractions in coeliac patients. Clin Sci (Lond). 66:357-64.

Ciclitira, P.J., Ellis, H.J., Wood, G.M., Howdle, P.D., and Losowsky, M.S. (1986). Secretion of gliadin antibody by coeliac jejunal mucosal biopsies cultured in vitro. Clin Exp Immunol. 64:119-24.

Clemente, M.G., De Virgiliis, S., Kang, J.S., Macatagney, R., Musu, M.P., Di Pierro, M.R., Drago, S., Congia, M., and Fasano, A. (2003). Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. Gut. 52:218-23.

Collin, P., Vilska, S., Heinonen, P.K., Hällström, O., and Pikkarainen, P. (1996). Infertility and coeliac disease. Gut. 39:382-4.

Daum, S., Cellier, C., and Mulder, C.J. (2005). Refractory coeliac disease. Best Pract Res Clin Gastroenterol. 19:413-24.

Daveson, A.J.M. (2009). Necator Americanus inhibits gluten sensitivity. A phase 2A randomized double blinded placebo controlled trial. Gut. 58:Suppl II:A59.

De Angelis, M., Rizzello, C.G., Fasano, A., Clemente, M.G., De Simone, C., Silano, M., De Vincenzi, M., Losito, I., and Gobbetti, M. (2006). VSL#3 probiotic preparation has the capacity to hydrolyze gliadin polypeptides responsible for Celiac Sprue. Biochim Biophys Acta. 1762:80-93.

de Kauwe, A.L., Chen, Z., Anderson, R.P., Keech, C.L., Price, J.D., Wijburg, O., Jackson, D.C., Ladhams, J., Allison, J., and McCluskey, J. (2009). Resistance to celiac disease in humanized HLA-DR3-DQ2-transgenic mice expressing specific anti-gliadin CD4+ T cells. J Immunol. 182:7440-50.

de Ritis, G., Auricchio, S., Jones, H.W., Lew, E.J., Bernardin, J.E., and Kasarda, D.D. (1988). In vitro (organ culture) studies of the toxicity of specific A-gliadin peptides in celiac disease. Gastroenterology. 94:41-9.

De Vincenzi, M., Dessi, M.R., Giovannini, C., Maialetti, F., and Mancini, E. (1995). Agglutinating activity of wheat gliadin peptide fractions in coeliac disease. Toxicology. 96:29-35.

Dessi, M., De Vincenzi, M., Maialetti, F., and Mancini, E. (1992). Effect of alpha-gliadin-derived peptides from bread and durum wheat on K562(S) cells. Ital J Gastroenterol. 24:397-9.

Dewar, D.H., Amato, M., Ellis, H.J., Pollock, E.L., Gonzalez-Cinca, N., Wieser, H., and Ciclitira, P.J. (2006). The toxicity of high molecular weight glutenin subunits of wheat to patients with coeliac disease. Eur J Gastroenterol Hepatol. 18:483-91.

Di Cagno, R., De Angelis, M., Auricchio, S., Greco, L., Clarke, C., De Vincenzi, M., Giovannini, C., D'Archivio, M., Landolfo, F., Parrilli, G., Minervini, F., Arendt, E., and Gobbetti, M. (2004). Sourdough bread made from wheat and nontoxic flours and started with selected lactobacilli is tolerated in celiac sprue patients. Appl Environ Microbiol. 70:1088-96.

Di Cagno, R., Rizzello, C.G., De Angelis, M., Cassone, A., Giuliani, G., Benedusi, A., Limitone, A., Surico, R.F., and Gobbetti, M. (2008). Use of selected sourdough strains of Lactobacillus for removing gluten and enhancing the nutritional properties of gluten-free bread. J Food Prot. 71:1491-5.

Di Niro, R., Sblattero, D., Florian, F., Stebel, M., Zentilin, L., Giacca, M., Villanacci, V., Galletti, A., Not, T., Ventura, A., and Marzari, R. (2008). Anti-idiotypic response in mice expressing human autoantibodies. Mol Immunol. 45:1782-91.

Di Sabatino, A., Ciccocioppo, R., Cupelli, F., Cinque, B., Millimaggi, D., Clarkson, M.M., Paulli, M., Cifone, M.G., and Corazza, G.R. (2006). Epithelium derived interleukin 15 regulates intraepithelial lymphocyte Th1 cytokine production, cytotoxicity, and survival in coeliac disease. Gut. 55:469-77.

Dicke, W.K. 1950. Coeliakie. University of Utrecht, Utrecht, The Netherlands.

Dicke, W.K., Weijers, H.A., and Van De Kamer, J.H. (1953). Coeliac disease. II. The presence in wheat of a factor having a deleterious effect in cases of coeliac disease. Acta Paediatr. 42:34-42.

Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., Riecken, E.O., and Schuppan, D. (1997). Identification of tissue transglutaminase as the autoantigen of celiac disease. Nat Med. 3:797-801.

Djilali-Saiah, I., Schmitz, J., Harfouch-Hammoud, E., Mougenot, J.F., Bach, J.F., and Caillat-Zucman, S. (1998). CTLA-4 gene polymorphism is associated with predisposition to coeliac disease. Gut. 43:187-9.

Dolfini, E., Elli, L., Dasdia, T., Bufardeci, B., Colleoni, M.P., Costa, B., Floriani, I., Falini, M.L., Guerrieri, N., Forlani, F., and Bardella, M.T. (2002). In vitro cytotoxic effect of bread wheat gliadin on the LoVo human adenocarcinoma cell line. Toxicol In Vitro. 16:331-7.

Dolfini, E., Elli, L., Ferrero, S., Braidotti, P., Roncoroni, L., Dasdia, T., Falini, M.L., Forlani, F., and Bardella, M.T. (2003). Bread wheat gliadin cytotoxicity: a new three-dimensional cell model. Scand J Clin Lab Invest. 63:135-41.

Dolfini, E., Roncoroni, L., Elli, L., Fumagalli, C., Colombo, R., Ramponi, S., Forlani, F., and Bardella, M.T. (2005a). Cytoskeleton reorganization and ultrastructural damage induced by gliadin in a three-dimensional in vitro model. World J Gastroenterol. 11:7597-601.

Dolfini, E., Elli, L., Roncoroni, L., Costa, B., Colleoni, M.P., Lorusso, V., Ramponi, S., Braidotti, P., Ferrero, S., Falini, M.L., and Bardella, M.T. (2005b). Damaging effects of gliadin on three-dimensional cell culture model. World J Gastroenterol. 11:5973-7.

Drago, S., El Asmar, R., Di Pierro, M., Grazia Clemente, M., Tripathi, A., Sapone, A., Thakar, M., Iacono, G., Carroccio, A., D'Agate, C., Not, T., Zampini, L., Catassi, C., and Fasano, A. (2006). Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. Scand J Gastroenterol. 41:408-19.

Dubois, P.C., Trynka, G., Franke, L., Hunt, K.A., Romanos, J., Curtotti, A., Zhernakova, A., Heap, G.A., Adany, R., Aromaa, A., Bardella, M.T., van den Berg, L.H., Bockett, N.A., de la Concha, E.G., Dema, B., Fehrmann, R.S., Fernandez-Arquero, M., Fiatal, S., Grandone, E., Green, P.M., Groen, H.J., Gwilliam, R., Houwen, R.H., Hunt, S.E., Kaukinen, K., Kelleher, D., Korponay-Szabo, I., Kurppa, K., MacMathuna, P., Mäki, M., Mazzilli, M.C., McCann, O.T., Mearin, M.L., Mein, C.A., Mirza, M.M., Mistry, V., Mora, B., Morley, K.I., Mulder, C.J., Murray, J.A., Nunez, C., Oosterom, E., Ophoff, R.A., Polanco, I., Peltonen, L., Platteel, M., Rybak, A., Salomaa, V., Schweizer, J.J.,

Sperandeo, M.P., Tack, G.J., Turner, G., Veldink, J.H., Verbeek, W.H., Weersma, R.K., Wolters, V.M., Urcelay, E., Cukrowska, B., Greco, L., Neuhausen, S.L., McManus, R., Barisani, D., Deloukas, P., Barrett, J.C., Saavalainen, P., Wijmenga, C., and van Heel, D.A. (2010). Multiple common variants for celiac disease influencing immune gene expression. Nat Genet. 42:295-302.

Ebert, E.C. (1998). Interleukin 15 is a potent stimulant of intraepithelial lymphocytes. Gastroenterology. 115:1439-45.

Ehren, J., Govindarajan, S., Moron, B., Minshull, J., and Khosla, C. (2008). Protein engineering of improved prolyl endopeptidases for celiac sprue therapy. Protein Eng Des Sel. 21:699-707.

Ehren, J., Moron, B., Martin, E., Bethune, M.T., Gray, G.M., and Khosla, C. (2009). A food-grade enzyme preparation with modest gluten detoxification properties. PLoS One. 4:e6313.

Ellis, H.J., Pollock, E.L., Engel, W., Fraser, J.S., Rosen-Bronson, S., Wieser, H., and Ciclitira, P.J. (2003). Investigation of the putative immunodominant T cell epitopes in coeliac disease. Gut. 52:212-7.

Esposito, C., Paparo, F., Caputo, I., Porta, R., Salvati, V.M., Mazzarella, G., Auricchio, S., and Troncone, R. (2003). Expression and enzymatic activity of small intestinal tissue transglutaminase in celiac disease. Am J Gastroenterol. 98:1813-20.

Fais, S., Maiuri, L., Pallone, F., De Vincenzi, M., De Ritis, G., Troncone, R., and Auricchio, S. (1992). Gliadin induced changes in the expression of MHC-class II antigens by human small intestinal epithelium. Organ culture studies with coeliac disease mucosa. Gut. 33:472-5.

Falchuk, Z.M., Gebhard, R.L., Sessoms, C., and Strober, W. (1974). An in vitro model of gluten-sensitive enteropathy. Effect of gliadin on intestinal epithelial cells of patients with gluten-sensitive enteropathy in organ culture. J Clin Invest. 53:487-500.

Falchuk, Z.M., Katz, A.J., Shwachman, H., Rogentine, G.N., and Strober, W. (1978). Gluten-sensitive enteropathy: genetic analysis and organ culture study in 35 families. Scand J Gastroenterol. 13:839-43.

Farquhar, M.G., and Palade, G.E. (1963). Junctional complexes in various epithelia. J Cell Biol. 17:375-412.

Farthing, M.J., Edwards, C.R., Rees, L.H., and Dawson, A.M. (1982). Male gonadal function in coeliac disease: 1. Sexual dysfunction, infertility, and semen quality. Gut. 23:608-14.

Fasano, A., Not, T., Wang, W., Uzzau, S., Berti, I., Tommasini, A., and Goldblum, S.E. (2000). Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. Lancet. 355:1518-9.

Ferguson, A., and Murray, D. (1971). Quantitation of intraepithelial lymphocytes in human jejunum. Gut. 12:988-94.

Fesus, L., and Piacentini, M. (2002). Transglutaminase 2: an enigmatic enzyme with diverse functions. Trends Biochem Sci. 27:534-9.

Fluge, G., and Aksnes, L. (1981). Morphological and morphometric assessment of human duodenal biopsies maintained in organ culture. In vitro influences of gluten in coeliac disease. Scand J Gastroenterol. 16:555-67.

Fluge, O., Sletten, K., Fluge, G., Aksnes, L., and Elsayed, S. (1994). In vitro toxicity of purified gluten peptides tested by organ culture. J Pediatr Gastroenterol Nutr. 18:186-92.

Freitag, T.L., Rietdijk, S., Junker, Y., Popov, Y., Bhan, A.K., Kelly, C.P., Terhorst, C., and Schuppan, D. (2009). Gliadin-primed CD4+CD45RBlowCD25- T cells drive gluten-dependent small intestinal damage after adoptive transfer into lymphopenic mice. Gut. 58:1597-605.

Friis, S., Dabelsteen, E., Sjöström, H., Noren, O., and Jarnum, S. (1992). Gliadin uptake in human enterocytes. Differences between coeliac patients in remission and control individuals. Gut. 33:1487-92.

Frisoni, M., Corazza, G., Lafiandra, D., De Ambrogio, E., Filipponi, C., Bonvicini, F., Borasio, E., Porceddu, E., and G, G. (1995). Wheat deficient in gliadins: promising tool for treatment of coeliac disease. Gut. 36:375-8.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., and Tsukita, S. (1993). Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol. 123:1777-88.

Furuse, M., Itoh, M., Hirase, T., Nagafuchi, A., Yonemura, S., and Tsukita, S. (1994). Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. J Cell Biol. 127:1617-26.

Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K., and Tsukita, S. (1998). Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol. 141:1539-50.

Galili, G., Altschuler, Y., and Levanony, H. (1993). Assembly and transport of seed storage proteins. Trends Cell Biol. 3:437-42.

Gass, J., Ehren, J., Strohmeier, G., Isaacs, I., and Khosla, C. (2005). Fermentation, purification, formulation, and pharmacological evaluation of a prolyl endopeptidase from Myxococcus xanthus: implications for Celiac Sprue therapy. Biotechnol Bioeng. 92:674-84.

Gass, J., Vora, H., Bethune, M.T., Gray, G.M., and Khosla, C. (2006). Effect of barley endoprotease EP-B2 on gluten digestion in the intact rat. J Pharmacol Exp Ther. 318:1178-86.

Gass, J., and Khosla, C. (2007). Prolyl endopeptidases. Cell Mol Life Sci. 64:345-55.

Gass, J., Bethune, M.T., Siegel, M., Spencer, A., and Khosla, C. (2007). Combination enzyme therapy for gastric digestion of dietary gluten in patients with celiac sprue. Gastroenterology. 133:472-80.

Gianfrani, C., Siciliano, R.A., Facchiano, A.M., Camarca, A., Mazzeo, M.F., Costantini, S., Salvati, V.M., Maurano, F., Mazzarella, G., Iaquinto, G., Bergamo, P., and Rossi, M. (2007). Transamidation of wheat flour inhibits the response to gliadin of intestinal T cells in celiac disease. Gastroenterology. 133:780-9.

Giovannini, C., Sanchez, M., Straface, E., Scazzocchio, B., Silano, M., and De Vincenzi, M. (2000). Induction of apoptosis in caco-2 cells by wheat gliadin peptides. Toxicology. 145:63-71.

Giovannini, C., Matarrese, P., Scazzocchio, B., Vari, R., D'Archivio, M., Straface, E., Masella, R., Malorni, W., and De Vincenzi, M. (2003). Wheat gliadin induces apoptosis of intestinal cells via an autocrine mechanism involving Fas-Fas ligand pathway. FEBS Lett. 540:117-24.

Gjertsen, H.A., Sollid, L.M., Ek, J., Thorsby, E., and Lundin, K.E. (1994). T cells from the peripheral blood of coeliac disease patients recognize gluten antigens when presented by HLA-DR, -DQ, or -DP molecules. Scand J Immunol. 39:567-74.

Gobbetti, M., Giuseppe Rizzello, C., Di Cagno, R., and De Angelis, M. (2007). Sourdough lactobacilli and celiac disease. Food Microbiol. 24:187-96.

Granzotto, M., dal Bo, S., Quaglia, S., Tommasini, A., Piscianz, E., Valencic, E., Ferrara, F., Martelossi, S., Ventura, A., and Not, T. (2009). Regulatory T-cell function is impaired in celiac disease. Dig Dis Sci. 54:1513-9.

Greco, L., Corazza, G., Babron, M.C., Clot, F., Fulchignoni-Lataud, M.C., Percopo, S., Zavattari, P., Bouguerra, F., Dib, C., Tosi, R., Troncone, R., Ventura, A., Mantavoni, W., Magazzu, G., Gatti, R., Lazzari, R., Giunta, A., Perri, F., Iacono, G., Cardi, E., de Virgiliis, S., Cataldo, F., De Angelis, G., Musumeci, S., Clerget-Darpoux, F., and et al. (1998). Genome search in celiac disease. Am J Hum Genet. 62:669-75.

Greco, L., Romino, R., Coto, I., Di Cosmo, N., Percopo, S., Maglio, M., Paparo, F., Gasperi, V., Limongelli, M.G., Cotichini, R., D'Agate, C., Tinto, N., Sacchetti, L., Tosi, R., and Stazi, M.A. (2002). The first large population based twin study of coeliac disease. Gut. 50:624-8.

Groschwitz, K.R., and Hogan, S.P. (2009). Intestinal barrier function: molecular regulation and disease pathogenesis. J Allergy Clin Immunol. 124:3-20; quiz 21-2.

Hadjivassiliou, M., Gibson, A., Davies-Jones, G.A., Lobo, A.J., Stephenson, T.J., and Milford-Ward, A. (1996). Does cryptic gluten sensitivity play a part in neurological illness? Lancet. 347:369-71.

Hadjivassiliou, M., Mäki, M., Sanders, D.S., Williamson, C.A., Grunewald, R.A., Woodroofe, N.M., and Korponay-Szabo, I.R. (2006). Autoantibody targeting of brain and intestinal transglutaminase in gluten ataxia. Neurology. 66:373-7.

Haimila, K., Smedberg, T., Mustalahti, K., Mäki, M., Partanen, J., and Holopainen, P. (2004). Genetic association of coeliac disease susceptibility to polymorphisms in the ICOS gene on chromosome 2q33. Genes Immun. 5:85-92.

Hall, H.J., Rubin, G., and Charnock, A. (2009). Systematic review: adherence to a gluten-free diet in adult patients with coeliac disease. Aliment Pharmacol Ther. 30:315-330.

Halttunen, T., and Mäki, M. (1999). Serum immunoglobulin A from patients with celiac disease inhibits human T84 intestinal crypt epithelial cell differentiation. Gastroenterology. 116:566-72.

Harris, O.D., Cooke, W.T., Thompson, H., and Waterhouse, J.A. (1967). Malignancy in adult coeliac disease and idiopathic steatorrhoea. Am J Med. 42:899-912.

Hartmann, G., Koehler, P., and Wieser, H. (2006). Rapid degradation of gliadin peptides toxic for celiac disease patients by proteases from germinating cereals. Journal of Cereal Science. 44:368-371.

Hauri, H.P., Kedinger, M., Haffen, K., Gaze, H., Hadorn, B., and Hekkens, W. (1978). Re-evaluation of the technique of organ culture for studying gluten toxicity in coeliac disease. Gut. 19:1090-8.

Hausch, F., Shan, L., Santiago, N.A., Gray, G.M., and Khosla, C. (2002). Intestinal digestive resistance of immunodominant gliadin peptides. Am J Physiol Gastrointest Liver Physiol. 283:G996-G1003.

Hershberg, R.M., and Mayer, L.F. (2000). Antigen processing and presentation by intestinal epithelial cells - polarity and complexity. Immunol Today. 21:123-8.

Hogberg, L., Laurin, P., Falth-Magnusson, K., Grant, C., Grodzinsky, E., Jansson, G., Ascher, H., Browaldh, L., Hammersjo, J.A., Lindberg, E., Myrdal, U., and Stenhammar, L. (2004). Oats to children with newly diagnosed coeliac disease: a randomised double blind study. Gut. 53:649-54.

Holm, K., Mäki, M., Vuolteenaho, N., Mustalahti, K., Ashorn, M., Ruuska, T., and Kaukinen, K. (2006). Oats in the treatment of childhood coeliac disease: a 2-year controlled trial and a long-term clinical follow-up study. Aliment Pharmacol Ther. 23:1463-72.

Howdle, P.D., Corazza, G.R., Bullen, A.W., and Losowsky, M.S. (1981). Gluten sensitivity of small intestinal mucosa in vitro: quantitative assessment of histologic change. Gastroenterology. 80:442-50.

Howdle, P.D., Ciclitira, P.J., Simpson, F.G., and Losowsky, M.S. (1984). Are all gliadins toxic in coeliac disease? An in vitro study of alpha, beta, gamma, and w gliadins. Scand J Gastroenterol. 19:41-7.

Hudson, D.A., Cornell, H.J., Purdham, D.R., and Rolles, C.J. (1976). Non-specific cytotoxicity of wheat gliadin components towards cultured human cells. Lancet. 1:339-41.

Hue, S., Mention, J.J., Monteiro, R.C., Zhang, S., Cellier, C., Schmitz, J., Verkarre, V., Fodil, N., Bahram, S., Cerf-Bensussan, N., and Caillat-Zucman, S. (2004). A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity. 21:367-77.

Hunt, K.A., Zhernakova, A., Turner, G., Heap, G.A., Franke, L., Bruinenberg, M., Romanos, J., Dinesen, L.C., Ryan, A.W., Panesar, D., Gwilliam, R., Takeuchi, F., McLaren, W.M., Holmes, G.K., Howdle, P.D., Walters, J.R., Sanders, D.S., Playford, R.J., Trynka, G., Mulder, C.J., Mearin, M.L., Verbeek, W.H., Trimble, V., Stevens, F.M., O'Morain, C., Kennedy, N.P., Kelleher, D., Pennington, D.J., Strachan, D.P., McArdle, W.L., Mein, C.A., Wapenaar, M.C., Deloukas, P., McGinnis, R., McManus,

R., Wijmenga, C., and van Heel, D.A. (2008). Newly identified genetic risk variants for celiac disease related to the immune response. Nat Genet. 40:395-402.
Izcue, A., Coombes, J.L., and Powrie, F. (2009). Regulatory lymphocytes and intestinal inflammation. Annu Rev Immunol. 27:313-38.
Jabri, B., de Serre, N.P., Cellier, C., Evans, K., Gache, C., Carvalho, C., Mougenot, J.F., Allez, M., Jian, R., Desreumaux, P., Colombel, J.F., Matuchansky, C., Cugnenc, H., Lopez-Botet, M., Vivier, E., Moretta, A., Roberts, A.I., Ebert, E.C., Guy-Grand, D., Brousse, N., Schmitz, J., and Cerf-Bensussan, N. (2000). Selective expansion of intraepithelial lymphocytes expressing the HLA-E-specific natural killer receptor CD94 in celiac disease. Gastroenterology. 118:867-79.
Jabri, B., and Sollid, L.M. (2009). Tissue-mediated control of immunopathology in coeliac disease. Nat Rev Immunol. 9:858-70.
Janatuinen, E.K., Pikkarainen, P.H., Kemppainen, T.A., Kosma, V.M., Järvinen, R.M., Uusitupa, M.I., and Julkunen, R.J. (1995). A comparison of diets with and without oats in adults with celiac disease. N Engl J Med. 333:1033-7.
Jelinkova, L., Tuckova, L., Cinova, J., Flegelova, Z., and Tlaskalova-Hogenova, H. (2004). Gliadin stimulates human monocytes to production of IL-8 and TNF-alpha through a mechanism involving NF-kappaB. FEBS Lett. 571:81-5.
Jones, B. (2005). Endoproteases of barley and malt. Journal of Cereal Science. 42:139-156.
Jos, J., Lenoir, G., Ritis, G.D., and Rey, J. (1975). In vitro pathogenetic studies of coeliac disease. Effects of protein digests on coeliac intestinal biopsy specimens maintained in culture for 48 hours. Scand J Gastroenterol. 10:121-8.
Jos, J., Labbe, F., Geny, B., and Griscelli, C. (1979). Immunoelectron-microscopic localization of immunoglobulin A and secretory component in jejunal mucosa from children with coeliac disease. Scand J Immunol. 9:441-50.
Kaeffer, B. (2002). Mammalian intestinal epithelial cells in primary culture: a mini-review. In Vitro Cell Dev Biol Anim. 38:123-34.
Kapoerchan, V.V., Wiesner, M., Hillaert, U., Drijfhout, J.W., Overhand, M., Alard, P., van der Marel, G.A., Overkleeft, H.S., and Koning, F. Design, synthesis and evaluation of high-affinity binders for the celiac disease associated HLA-DQ2 molecule. Mol Immunol. 47:1091-7.
Karpati, S., Kosnai, I., Torok, E., and Kovacs, J.B. (1988). Immunoglobulin A deposition in jejunal mucosa of children with dermatitis herpetiformis. J Invest Dermatol. 91:336-9.
Kasarda, D.D., Bernardin, J.E., and Qualset, C.O. (1976). Relationship of gliadin protein components to chromosomes in hexaploid wheats (Triticum aestivum L.). Proc Natl Acad Sci U S A. 73:3646-3650.
Kasarda, D.D., Okita, T.W., Bernardin, J.E., Preston, B.A., Nimmo, C.C., Lew, E.J.L., Dietler, M.D., and Greene, F.C. (1984). Nucleic acid (cDNA) and amino acid sequences of a-type gliadins from wheat (*Triticum aestivum*). Proc Natl Acad Sci. 81:4712-6.
Kasarda, D.D. (1997). Gluten and gliadin: precipitating factors in coeliac disease. *In* Coeliac Disease: Proceedings of the 7th International Symposium on Coeliac Disease. M. Mäki, P. Collin, and J.K. Visakorpi, editors, Coeliac Disease Study Group, Institute of Medical Techology, Tampere, Finland. 195-212.
Kaukinen, K., Halme, L., Collin, P., Farkkila, M., Mäki, M., Vehmanen, P., Partanen, J., and Höckerstedt, K. (2002). Celiac disease in patients with severe liver disease: gluten-free diet may reverse hepatic failure. Gastroenterology. 122:881-8.
Kaukinen, K., Peräaho, M., Collin, P., Partanen, J., Woolley, N., Kaartinen, T., Nuutinen, T., Halttunen, T., Mäki, M., and Korponay-Szabo, I. (2005). Small-bowel mucosal transglutaminase 2-specific IgA deposits in coeliac disease without villous atrophy: A prospective and randomized clinical study. Scand J Gastroenterol. 40:564-72.
Keech, C.L., Dromey, J., Chen, Z., Anderson, R.P., and McCluskey, J. (2009). Immune tolerance induced by peptide immunotheraphy in an HLA-DQ2-dependent mouse model of gluten immunity. Gastroenterology. 136:Suppl I:A57.
Kendall, M.J., Schneider, R., Cox, P.S., and Hawkins, C.F. (1972). Gluten subfractions in coeliac disease. Lancet. 2:1065-7.

Kieffer, M., Frazier, P.J., Daniels, N.W., and Coombs, R.R. (1982). Wheat gliadin fractions and other cereal antigens reactive with antibodies in the sera of coeliac patients. Clin Exp Immunol. 50:651-60.

Kilmartin, C., Wieser, H., Abuzakouk, M., Kelly, J., Jackson, J., and Feighery, C. (2006). Intestinal T cell responses to cereal proteins in celiac disease. Dig Dis Sci. 51:202-9.

Korponay-Szabo, I.R., Sulkanen, S., Halttunen, T., Maurano, F., Rossi, M., Mazzarella, G., Laurila, K., Troncone, R., and Mäki, M. (2000). Tissue transglutaminase is the target in both rodent and primate tissues for celiac disease-specific autoantibodies. J Pediatr Gastroenterol Nutr. 31:520-7.

Korponay-Szabo, I.R., Halttunen, T., Szalai, Z., Laurila, K., Kiraly, R., Kovacs, J.B., Fesus, L., and Mäki, M. (2004). In vivo targeting of intestinal and extraintestinal transglutaminase 2 by coeliac autoantibodies. Gut. 53:641-8.

Koskinen, L.L., Korponay-Szabo, I.R., Viiri, K., Juuti-Uusitalo, K., Kaukinen, K., Lindfors, K., Mustalahti, K., Kurppa, K., Adany, R., Pocsai, Z., Szeles, G., Einarsdottir, E., Wijmenga, C., Mäki, M., Partanen, J., Kere, J., and Saavalainen, P. (2008). Myosin IXB gene region and gluten intolerance: linkage to coeliac disease and a putative dermatitis herpetiformis association. J Med Genet. 45:222-7.

Koskinen, O., Collin, P., Lindfors, K., Laurila, K., Mäki, M., and Kaukinen, K. (2009). Usefulness of Small-bowel Mucosal Transglutaminase-2 Specific Autoantibody Deposits in the Diagnosis and Follow-up of Celiac Disease. J Clin Gastroenterol.

Kuitunen, P., Kosnai, I., and Savilahti, E. (1982). Morphometric study of the jejunal mucosa in various childhood enteropathies with special reference to intraepithelial lymphocytes. J Pediatr Gastroenterol Nutr. 1:525-31.

Kutlu, T., Brousse, N., Rambaud, C., Le Deist, F., Schmitz, J., and Cerf-Bensussan, N. (1993). Numbers of T cell receptor (TCR) alpha beta+ but not of TcR gamma delta+ intraepithelial lymphocytes correlate with the grade of villous atrophy in coeliac patients on a long term normal diet. Gut. 34:208-14.

Ladinser, B., Rossipal, E., and Pittschieler, K. (1994). Endomysium antibodies in coeliac disease: an improved method. Gut. 35:776-8.

Laitila, A., Kotaviita, E., Peltola, P., Home, S., and Wilhelmson, A. (2007). Indigenous microbial community of barley greatly influences grain germination and malt quality. J. Inst. Brew. 113:9-20.

Lammers, K.M., Lu, R., Brownley, J., Lu, B., Gerard, C., Thomas, K., Rallabhandi, P., Shea-Donohue, T., Tamiz, A., Alkan, S., Netzel-Arnett, S., Antalis, T., Vogel, S.N., and Fasano, A. (2008). Gliadin induces an increase in intestinal permeability and zonulin release by binding to the chemokine receptor CXCR3. Gastroenterology. 135:194-204 e3.

Li, N., DeMarco, V.G., West, C.M., and Neu, J. (2003). Glutamine supports recovery from loss of transepithelial resistance and increase of permeability induced by media change in Caco-2 cells. J Nutr Biochem. 14:401-8.

Lindfors, K., Blomqvist, T., Juuti-Uusitalo, K., Stenman, S., Venäläinen, J., Mäki, M., and Kaukinen, K. (2008). Live probiotic Bifidobacterium lactis bacteria inhibit the toxic effects induced by wheat gliadin in epithelial cell culture. Clin Exp Immunol. 152:552-8.

Lindfors, K., Kaukinen, K. and Mäki M. (2009). A role for anti-transglutaminase 2 autoantibodies in the pathogenesis of coeliac disease? Amino Acids. 36:685-91.

Liu, J., Juo, S.H., Holopainen, P., Terwilliger, J., Tong, X., Grunn, A., Brito, M., Green, P., Mustalahti, K., Mäki, M., Gilliam, T.C., and Partanen, J. (2002). Genomewide linkage analysis of celiac disease in Finnish families. Am J Hum Genet. 70:51-9.

Logan, R.F., Tucker, G., Rifkind, E.A., Heading, R.C., and Ferguson, A. (1983). Changes in clinical features of coeliac disease in adults in Edinburgh and the Lothians 1960-79. Br Med J (Clin Res Ed). 286:95-7.

Lohi, S., Mustalahti, K., Kaukinen, K., Laurila, K., Collin, P., Rissanen, H., Lohi, O., Bravi, E., Gasparin, M., Reunanen, A., and Mäki, M. (2007). Increasing prevalence of coeliac disease over time. Aliment Pharmacol Ther. 26:1217-25.

Loponen, J., Sontag-Strohm, T., Venäläinen, J., and Salovaara, H. (2007). Prolamin hydrolysis in wheat sourdoughs with differing proteolytic activities. J Agric Food Chem. 55:978-984.

Loponen, J., Kanerva, P., Zhang, C., Sontag-Strohm, T., Salovaara, H., and Gänzle, M. (2009). Prolamin hydrolysis and pentosan solubilization in germinated-rye sourdoughs determined by chromatograph and immunological methods. J Agric Food Chem. 57:746-753.

Lundin, K.E., Scott, H., Hansen, T., Paulsen, G., Halstensen, T.S., Fausa, O., Thorsby, E., and Sollid, L.M. (1993). Gliadin-specific, HLA-DQ(alpha 1*0501,beta 1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. J Exp Med. 178:187-96.

Lundin, K.E., Sollid, L.M., Anthonsen, D., Noren, O., Molberg, O., Thorsby, E., and Sjöström, H. (1997). Heterogeneous reactivity patterns of HLA-DQ-restricted, small intestinal T-cell clones from patients with celiac disease. Gastroenterology. 112:752-9.

Luostarinen, L., Pirttilä, T., and Collin, P. (1999). Coeliac disease presenting with neurological disorders. Eur Neurol. 42:132-5.

Madara, J.L., Stafford, J., Dharmsathaphorn, K., and Carlson, S. (1987). Structural analysis of a human intestinal epithelial cell line. Gastroenterology. 92:1133-45.

Maiuri, L., Picarelli, A., Boirivant, M., Coletta, S., Mazzilli, M.C., De Vincenzi, M., Londei, M., and Auricchio, S. (1996). Definition of the initial immunologic modifications upon in vitro gliadin challenge in the small intestine of celiac patients. Gastroenterology. 110:1368-78.

Maiuri, L., Auricchio, S., Coletta, S., De Marco, G., Picarelli, A., Di Tola, M., Quaratino, S., and Londei, M. (1998). Blockage of T-cell costimulation inhibits T-cell action in celiac disease. Gastroenterology. 115:564-72.

Maiuri, L., Ciacci, C., Auricchio, S., Brown, V., Quaratino, S., and Londei, M. (2000). Interleukin 15 mediates epithelial changes in celiac disease. Gastroenterology. 119:996-1006.

Maiuri, L., Ciacci, C., Vacca, L., Ricciardelli, I., Auricchio, S., Quaratino, S., and Londei, M. (2001a). IL-15 drives the specific migration of CD94+ and TCR-gammadelta+ intraepithelial lymphocytes in organ cultures of treated celiac patients. Am J Gastroenterol. 96:150-6.

Maiuri, L., Ciacci, C., Raia, V., Vacca, L., Ricciardelli, I., Raimondi, F., Auricchio, S., Quaratino, S., and Londei, M. (2001b). FAS engagement drives apoptosis of enterocytes of coeliac patients. Gut. 48:418-24.

Maiuri, L., Ciacci, C., Ricciardelli, I., Vacca, L., Raia, V., Auricchio, S., Picard, J., Osman, M., Quaratino, S., and Londei, M. (2003). Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. Lancet. 362:30-7.

Maiuri, L., Ciacci, C., Ricciardelli, I., Vacca, L., Raia, V., Rispo, A., Griffin, M., Issekutz, T., Quaratino, S., and Londei, M. (2005). Unexpected role of surface transglutaminase type II in celiac disease. Gastroenterology. 129:1400-13.

Mäki, M., Kallonen, K., Lähdeaho, M.L., and Visakorpi, J.K. (1988). Changing pattern of childhood coeliac disease in Finland. Acta Paediatr Scand. 77:408-12.

Mäki, M., Holm, K., Lipsanen, V., Hällström, O., Viander, M., Collin, P., Savilahti, E., and Koskimies, S. (1991). Serological markers and HLA genes among healthy first-degree relatives of patients with coeliac disease. Lancet. 338:1350-3.

Mäki, M., Mustalahti, K., Kokkonen, J., Kulmala, P., Haapalahti, M., Karttunen, T., Ilonen, J., Laurila, K., Dahlbom, I., Hansson, T., Hopfl, P., and Knip, M. (2003). Prevalence of Celiac disease among children in Finland. N Engl J Med. 348:2517-24.

Marietta, E., Black, K., Camilleri, M., Krause, P., Rogers, R.S., 3rd, David, C., Pittelkow, M.R., and Murray, J.A. (2004). A new model for dermatitis herpetiformis that uses HLA-DQ8 transgenic NOD mice. J Clin Invest. 114:1090-7.

Marks, J., Shuster, S., and Watson, A.J. (1966). Small-bowel changes in dermatitis herpetiformis. Lancet. 2:1280-2.

Marsh, M.N. (1992). Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). Gastroenterology. 102:330-54.

Marti, T., Molberg, O., Li, Q., Gray, G.M., Khosla, C., and Sollid, L.M. (2005). Prolyl endopeptidase-mediated destruction of T cell epitopes in whole gluten: chemical and immunological characterization. J Pharmacol Exp Ther. 312:19-26.

Martin-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., Panzeri, C., Stoppacciaro, A., Ruco, L., Villa, A., Simmons, D., and Dejana, E. (1998). Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol. 142:117-27.

Martin-Pagola, A., Perez-Nanclares, G., Ortiz, L., Vitoria, J.C., Hualde, I., Zaballa, R., Preciado, E., Castano, L., and Bilbao, J.R. (2004). MICA response to gliadin in intestinal mucosa from celiac patients. Immunogenetics. 56:549-54.

Martucci, S., Fraser, J.S., Biagi, F., Corazza, G.R., Ciclitira, P.J., and Ellis, H.J. (2003). Characterizing one of the DQ2 candidate epitopes in coeliac disease: A-gliadin 51-70 toxicity assessed using an organ culture system. Eur J Gastroenterol Hepatol. 15:1293-8.

Marzari, R., Sblattero, D., Florian, F., Tongiorgi, E., Not, T., Tommasini, A., Ventura, A., and Bradbury, A. (2001). Molecular dissection of the tissue transglutaminase autoantibody response in celiac disease. J Immunol. 166:4170-6.

Matysiak-Budnik, T., Candalh, C., Dugave, C., Namane, A., Cellier, C., Cerf-Bensussan, N., and Heyman, M. (2003). Alterations of the intestinal transport and processing of gliadin peptides in celiac disease. Gastroenterology. 125:696-707.

Matysiak-Budnik, T., Candalh, C., Cellier, C., Dugave, C., Namane, A., Vidal-Martinez, T., Cerf-Bensussan, N., and Heyman, M. (2005). Limited efficiency of prolyl-endopeptidase in the detoxification of gliadin peptides in celiac disease. Gastroenterology. 129:786-96.

Matysiak-Budnik, T., Moura, I.C., Arcos-Fajardo, M., Lebreton, C., Menard, S., Candalh, C., Ben-Khalifa, K., Dugave, C., Tamouza, H., van Niel, G., Bouhnik, Y., Lamarque, D., Chaussade, S., Malamut, G., Cellier, C., Cerf-Bensussan, N., Monteiro, R.C., and Heyman, M. (2008). Secretory IgA mediates retrotranscytosis of intact gliadin peptides via the transferrin receptor in celiac disease. J Exp Med. 205:143-54.

Maurano, F., Siciliano, R.A., De Giulio, B., Luongo, D., Mazzeo, M.F., Troncone, R., Auricchio, S., and Rossi, M. (2001). Intranasal administration of one alpha gliadin can downregulate the immune response to whole gliadin in mice. Scand J Immunol. 53:290-5.

Mazzarella, G., Maglio, M., Paparo, F., Nardone, G., Stefanile, R., Greco, L., van de Wal, Y., Kooy, Y., Koning, F., Auricchio, S., and Troncone, R. (2003). An immunodominant DQ8 restricted gliadin peptide activates small intestinal immune response in in vitro cultured mucosa from HLA-DQ8 positive but not HLA-DQ8 negative coeliac patients. Gut. 52:57-62.

Mazzarella, G., Stefanile, R., Camarca, A., Giliberti, P., Cosentini, E., Marano, C., Iaquinto, G., Giardullo, N., Auricchio, S., Sette, A., Troncone, R., and Gianfrani, C. (2008). Gliadin activates HLA class I-restricted CD8+ T cells in celiac disease intestinal mucosa and induces the enterocyte apoptosis. Gastroenterology. 134:1017-27.

Mention, J.J., Ben Ahmed, M., Begue, B., Barbe, U., Verkarre, V., Asnafi, V., Colombel, J.F., Cugnenc, P.H., Ruemmele, F.M., McIntyre, E., Brousse, N., Cellier, C., and Cerf-Bensussan, N. (2003). Interleukin 15: a key to disrupted intraepithelial lymphocyte homeostasis and lymphomagenesis in celiac disease. Gastroenterology. 125:730-45.

Meresse, B., Chen, Z., Ciszewski, C., Tretiakova, M., Bhagat, G., Krausz, T.N., Raulet, D.H., Lanier, L.L., Groh, V., Spies, T., Ebert, E.C., Green, P.H., and Jabri, B. (2004). Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. Immunity. 21:357-66.

Meresse, B., Curran, S.A., Ciszewski, C., Orbelyan, G., Setty, M., Bhagat, G., Lee, L., Tretiakova, M., Semrad, C., Kistner, E., Winchester, R.J., Braud, V., Lanier, L.L.,

Geraghty, D.E., Green, P.H., Guandalini, S., and Jabri, B. (2006). Reprogramming of CTLs into natural killer-like cells in celiac disease. J Exp Med. 203:1343-55.
Messer, M., Anderson, C.M., and Hubbard, L. (1964). Studies on the Mechanism of Destruction of the Toxic Action of Wheat Gluten in Coeliac Disease by Crude Papain. Gut. 5:295-303.
M'Hir, S., Rizzello, C.G., Di Cagno, R., Cassone, A., and Hamdi, M. (2009). Use of selected enterococci and Rhizopus oryzae proteases to hydrolyse wheat proteins responsible for celiac disease. J Appl Microbiol. 106:421-31.
Mikola, L. (1986). Acid carboxypeptidases in grains and leaves of wheat, *Triticum aestivum*. Plant Physiol. 81:823-829.
Mitea, C., Havenaar, R., Drijfhout, J.W., Edens, L., Dekking, L., and Koning, F. (2008). Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: implications for coeliac disease. Gut. 57:25-32.
Molberg, O., McAdam, S.N., Korner, R., Quarsten, H., Kristiansen, C., Madsen, L., Fugger, L., Scott, H., Noren, O., Roepstorff, P., Lundin, K.E., Sjöström, H., and Sollid, L.M. (1998). Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. Nat Med. 4:713-7.
Molberg, O., McAdam, S.N., Lundin, K.E., and Sollid, L.M. (2000). Studies of gliadin-specific T cells in celiac disease. *In* Celiac Disease: Methods and Protocols. M.N. Marsh, editor. Humana Press, Totowa. 105.
Molberg, O., Solheim Flaete, N., Jensen, T., Lundin, K.E., Arentz-Hansen, H., Anderson, O.D., Kjersti Uhlen, A., and Sollid, L.M. (2003). Intestinal T-cell responses to high-molecular-weight glutenins in celiac disease. Gastroenterology. 125:337-44.
Monsuur, A.J., de Bakker, P.I., Alizadeh, B.Z., Zhernakova, A., Bevova, M.R., Strengman, E., Franke, L., van't Slot, R., van Belzen, M.J., Lavrijsen, I.C., Diosdado, B., Daly, M.J., Mulder, C.J., Mearin, M.L., Meijer, J.W., Meijer, G.A., van Oort, E., Wapenaar, M.C., Koeleman, B.P., and Wijmenga, C. (2005). Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. Nat Genet. 37:1341-4.
Montalto, M., Cuoco, L., Ricci, R., Maggiano, N., Vecchio, F.M., and Gasbarrini, G. (2002). Immunohistochemical analysis of ZO-1 in the duodenal mucosa of patients with untreated and treated celiac disease. Digestion. 65:227-33.
Mulder, C.J., Wahab, P.J., Meijer, J.W., and Metselaar, E. (2001). A pilot study of recombinant human interleukin-10 in adults with refractory coeliac disease. Eur J Gastroenterol Hepatol. 13:1183-8.
Mustalahti, K., Collin, P., Sievänen, H., Salmi, J., and Mäki, M. (1999). Osteopenia in patients with clinically silent coeliac disease warrants screening. Lancet. 354:744-5.
Myrsky, E., Kaukinen, K., Syrjänen, M., Korponay-Szabo, I., Mäki, M., and Lindfors, K. (2008). Coeliac disease-specific autoantibodies targeted against transglutaminase-2 disturb angiogenesis. Clin Exp Immunol. 152:111-119.
Myrsky, E., Caja, S., Simon-Vecsei, Z., Korponay-Szabo, I., Nadalutti, C., Collinghan, R., Mongeot, A., Griffin, M., Mäki, M., Kaukinen, K., and Lindfors, K. (2009). Coeliac disease IgA modulates vascular permeability in vitro through the activity of transglutaminase-2 and RhoA. Cell Mol Life Sci. 66:3375-85.
Myöhänen, T.T., Venäläinen, J.I., Tupala, E., Garcia-Horsman, J.A., Miettinen, R., and Mannisto, P.T. (2007). Distribution of immunoreactive prolyl oligopeptidase in human and rat brain. Neurochem Res. 32:1365-74.
Nilsen, E.M., Lundin, K.E., Krajci, P., Scott, H., Sollid, L.M., and Brandtzaeg, P. (1995). Gluten specific, HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Th1 or Th0 profile dominated by interferon gamma. Gut. 37:766-76.
Nilsen, E.M., Gjertsen, H.A., Jensen, K., Brandtzaeg, P., and Lundin, K.E. (1996). Gluten activation of peripheral blood T cells induces a Th0-like cytokine pattern in both coeliac patients and controls. Clin Exp Immunol. 103:295-303.
Osborne, T.B. 1907. The protein of the wheat kernel. Carnegie Institute of Washington, Washington DC, USA.

Palova-Jelinkova, L., Rozkova, D., Pecharova, B., Bartova, J., Sediva, A., Tlaskalova-Hogenova, H., Spisek, R., and Tuckova, L. (2005). Gliadin fragments induce phenotypic and functional maturation of human dendritic cells. J Immunol. 175:7038-45.

Paterson, B.M., Lammers, K.M., Arrieta, M.C., Fasano, A., and Meddings, J.B. (2007). The safety, tolerance, pharmacokinetic and pharmacodynamic effects of single doses of AT-1001 in coeliac disease subjects: a proof of concept study. Aliment Pharmacol Ther. 26:757-66.

Petronzelli, F., Bonamico, M., Ferrante, P., Grillo, R., Mora, B., Mariani, P., Apollonio, I., Gemme, G., and Mazzilli, M.C. (1997). Genetic contribution of the HLA region to the familial clustering of coeliac disease. Ann Hum Genet. 61:307-17.

Picarelli, A., Maiuri, L., Frate, A., Greco, M., Auricchio, S., and Londei, M. (1996). Production of antiendomysial antibodies after in-vitro gliadin challenge of small intestine biopsy samples from patients with coeliac disease. Lancet. 348:1065-7.

Picarelli, A., Di Tola, M., Sabbatella, L., Anania, M.C., Di Cello, T., Greco, R., Silano, M., and De Vincenzi, M. (1999). 31-43 amino acid sequence of the alpha-gliadin induces anti-endomysial antibody production during in vitro challenge. Scand J Gastroenterol. 34:1099-102.

Picarelli, A., Sabbatella, L., Di Tola, M., Vetrano, S., Maffia, C., Picchi, C., Mastracchio, A., Paoluzi, P., and Anania, M.C. (2001). Forty-eight hours of biopsy culture improve the sensitivity of the in vitro gliadin challenge in the diagnosis of celiac disease. Clin Chem. 47:1841-3.

Picarelli, A., Di Tola, M., Sabbatella, L., Anania, M.C., Calabro, A., Renzi, D., Bai, J.C., Sugai, E., Carroccio, A., Di Prima, L., Bardella, M.T., Barisani, D., Ribes-Koninckx, C., Aliaga, E.D., Gasparin, M., and Bravi, E. (2006). Usefulness of the organ culture system in the in vitro diagnosis of coeliac disease: a multicentre study. Scand J Gastroenterol. 41:186-90.

Piper, J.L., Gray, G.M., and Khosla, C. (2004). Effect of prolyl endopeptidase on digestive-resistant gliadin peptides in vivo. J Pharmacol Exp Ther. 311:213-9.

Pizzuti, D., Bortolami, M., Mazzon, E., Buda, A., Guariso, G., D'Odorico, A., Chiarelli, S., D'Inca, R., De Lazzari, F., and Martines, D. (2004). Transcriptional downregulation of tight junction protein ZO-1 in active coeliac disease is reversed after a gluten-free diet. Dig Liver Dis. 36:337-41.

Pizzuti, D., Buda, A., D'Odorico, A., D'Inca, R., Chiarelli, S., Curioni, A., and Martines, D. (2006). Lack of intestinal mucosal toxicity of Triticum monococcum in celiac disease patients. Scand J Gastroenterol. 41:1305-11.

Polgar, L. (2002). The prolyl oligopeptidase family. Cell Mol Life Sci. 59:349-62.

Polvi, A., Eland, C., Koskimies, S., Mäki, M., and Partanen, J. (1996). HLA DQ and DP in Finnish families with celiac disease. Eur J Immunogenet. 23:221-34.

Pyle, G.G., Paaso, B., Anderson, B.E., Allen, D.D., Marti, T., Li, Q., Siegel, M., Khosla, C., and Gray, G.M. (2005). Effect of pretreatment of food gluten with prolyl endopeptidase on gluten-induced malabsorption in celiac sprue. Clin Gastroenterol Hepatol. 3:687-94.

Qiao, S.W., Bergseng, E., Molberg, O., Xia, J., Fleckenstein, B., Khosla, C., and Sollid, L.M. (2004). Antigen presentation to celiac lesion-derived T cells of a 33-mer gliadin peptide naturally formed by gastrointestinal digestion. J Immunol. 173:1757-62.

Raki, M., Tollefsen, S., Molberg, O., Lundin, K.E., Sollid, L.M., and Jahnsen, F.L. (2006). A unique dendritic cell subset accumulates in the celiac lesion and efficiently activates gluten-reactive T cells. Gastroenterology. 131:428-38.

Raki, M., Fallang, L.E., Brottveit, M., Bergseng, E., Quarsten, H., Lundin, K.E., and Sollid, L.M. (2007). Tetramer visualization of gut-homing gluten-specific T cells in the peripheral blood of celiac disease patients. Proc Natl Acad Sci U S A. 104:2831-6.

Reinke, Y., Zimmer, K.P., and Naim, H.Y. (2009). Toxic peptides in Frazer's fraction interact with the actin cytoskeleton and affect the targeting and function of intestinal proteins. Exp Cell Res. 315:3442-52.

Risdon, R.A., and Keeling, J.W. (1974). Quantitation of the histological changes found in small intestinal biopsy specimens from children with suspected coeliac disease. Gut. 15:9-18.

Rivabene, R., Mancini, E., and De Vincenzi, M. (1999). In vitro cytotoxic effect of wheat gliadin-derived peptides on the Caco-2 intestinal cell line is associated with intracellular oxidative imbalance: implications for coeliac disease. Biochim Biophys Acta. 1453:152-60.

Rizzello, C.G., De Angelis, M., Di Cagno, R., Camarca, A., Silano, M., Losito, I., De Vincenzi, M., De Bari, M.D., Palmisano, F., Maurano, F., Gianfrani, C., and Gobbetti, M. (2007). Highly efficient gluten degradation by lactobacilli and fungal proteases during food processing: new perspectives for celiac disease. Appl Environ Microbiol. 73:4499-507.

Rocca, E., Paganuzzi Stammati, A., Zampaglioni, F., and Zucco, F. (1983). Effects of gliadin-derived peptides from bread and durum wheats on in vitro cultures of human cell lines. Implications for coeliac disease pathogenesis. Toxicol Lett. 16:331-8.

Rocher, A., Calero, M., Soriano, F., and Mendez, E. (1996). Identification of major rye secalins as coeliac immunoreactive proteins. Biochim Biophys Acta. 1295:13-22.

Rubin, C.E., Brandborg, L.L., Flick, A.L., Phelps, P., Parmentier, C., and Van Niel, S. (1962). Studies of celiac sprue. III. The effect of repeated wheat instillation into the proximal ileum of patients on a gluten free diet. Gastroenterology. 43:621-41.

Sakly, W., Thomas, V., Quash, G., and El Alaoui, S. (2006). A role for tissue transglutaminase in alpha-gliadin peptide cytotoxicity. Clin Exp Immunol. 146:550-8.

Salmi, T.T., Collin, P., Järvinen, O., Haimila, K., Partanen, J., Laurila, K., Korponay-Szabo, I.R., Huhtala, H., Reunala, T., Mäki, M., and Kaukinen, K. (2006a). Immunoglobulin A autoantibodies against transglutaminase 2 in the small intestinal mucosa predict forthcoming coeliac disease. Aliment Pharmacol Ther. 24:541-52.

Salmi, T.T., Collin, P., Korponay-Szabo, I.R., Laurila, K., Partanen, J., Huhtala, H., Kiraly, R., Lorand, L., Reunala, T., Mäki, M., and Kaukinen, K. (2006b). Endomysial antibody-negative coeliac disease: clinical characteristics and intestinal autoantibody deposits. Gut.

Salvati, V.M., Mazzarella, G., Gianfrani, C., Levings, M.K., Stefanile, R., De Giulio, B., Iaquinto, G., Giardullo, N., Auricchio, S., Roncarolo, M.G., and Troncone, R. (2005). Recombinant human interleukin 10 suppresses gliadin dependent T cell activation in ex vivo cultured coeliac intestinal mucosa. Gut. 54:46-53.

Sander, G.R., Cummins, A.G., Henshall, T., and Powell, B.C. (2005). Rapid disruption of intestinal barrier function by gliadin involves altered expression of apical junctional proteins. FEBS Lett. 579:4851-5.

Sato, T., Vries, RG., Snippert, HJ., van de Watering, M., Barker, N., Stange, DE., van Es, JH., Abo, A., Kujala, P., Peters, PJ. and Clevers H. (2009). Single Lgr5 stem cells build crypt-villous structures in vitro without a mesenchymal niche. Nature. 459(7244):262-5.

Schneeberger, E.E., and Lynch, R.D. (2004). The tight junction: a multifunctional complex. Am J Physiol Cell Physiol. 286:C1213-28.

Schulzke, J.D., Schulzke, I., Fromm, M., and Riecken, E.O. (1995). Epithelial barrier and ion transport in coeliac sprue: electrical measurements on intestinal aspiration biopsy specimens. Gut. 37:777-82.

Schulzke, J.D., Bentzel, C.J., Schulzke, I., Riecken, E.O., and Fromm, M. (1998). Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. Pediatr Res. 43:435-41.

Schumann, M., Richter, J.F., Wedell, I., Moos, V., Zimmermann-Kordmann, M., Schneider, T., Daum, S., Zeitz, M., Fromm, M., and Schulzke, J.D. (2008). Mechanisms of epithelial translocation of the alpha(2)-gliadin-33mer in coeliac sprue. Gut. 57:747-54.

Schwertz, E., Kahlenberg, F., Sack, U., Richter, T., Stern, M., Conrad, K., Zimmer, K.P., and Mothes, T. (2004). Serologic assay based on gliadin-related nonapeptides as a highly sensitive and specific diagnostic aid in celiac disease. Clin Chem. 50:2370-5.

Seah, P.P., Fry, L., Rossiter, M.A., Hoffbrand, A.V., and Holborow, E.J. (1971). Anti-reticulin antibodies in childhood coeliac disease. Lancet. 2:681-2.

Senger, S., Luongo, D., Maurano, F., Mazzeo, M.F., Siciliano, R.A., Gianfrani, C., David, C., Troncone, R., Auricchio, S., and Rossi, M. (2003). Intranasal administration of a recombinant alpha-gliadin down-regulates the immune response to wheat gliadin in DQ8 transgenic mice. Immunol Lett. 88:127-34.

Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G.M., Sollid, L.M., and Khosla, C. (2002). Structural basis for gluten intolerance in celiac sprue. Science. 297:2275-9.

Shan, L., Marti, T., Sollid, L.M., Gray, G.M., and Khosla, C. (2004). Comparative biochemical analysis of three bacterial prolyl endopeptidases: implications for coeliac sprue. Biochem J. 383:311-8.

Shan, L., Qiao, S.W., Arentz-Hansen, H., Molberg, O., Gray, G.M., Sollid, L.M., and Khosla, C. (2005). Identification and analysis of multivalent proteolytically resistant peptides from gluten: implications for celiac sprue. J Proteome Res. 4:1732-41.

Shewry, P.R., Napier, J.A., and Tatham, A.S. (1995). Seed Storage Proteins: Structure and Biosynthesis. The Plant Cell. 7:945-956.

Shewry, P.R., Tatham, A.S., and Halford, N.G. (1999). The prolamins of *Triticeae*. *In* Seed proteins. P.R. Shewry and R. Casey, editors. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Shewry, P.R., Popineau, Y., Lafiandra, D., and Belton, P. (2001). Wheat glutenin subunits and dough elasticity: findings ot the EUROWHEAT project. Trends in Food Science and Technology. 11:433-441.

Shidrawi, R.G., Day, P., Przemioslo, R., Ellis, H.J., Nelufer, J.M., and Ciclitira, P.J. (1995). In vitro toxicity of gluten peptides in coeliac disease assessed by organ culture. Scand J Gastroenterol. 30:758-63.

Siegel, M., Bethune, M.T., Gass, J., Ehren, J., Xia, J., Johannsen, A., Stuge, T.B., Gray, G.M., Lee, P.P., and Khosla, C. (2006). Rational design of combination enzyme therapy for celiac sprue. Chem Biol. 13:649-58.

Siegel, M., Xia, J., and Khosla, C. (2007). Structure-based design of alpha-amido aldehyde containing gluten peptide analogues as modulators of HLA-DQ2 and transglutaminase 2. Bioorg Med Chem. 15:6253-61.

Silano, M., Di Banedetto, R., Trecca, A., Arrabito, G., Leonardi, F., and De Vincenzi, M. (2007). A decapeptide from durum wheat prevents celiac peripheral blood lymphocytes from activation by gliadin peptides. Pediatr Res. 61:67-71.

Silano, M., Di Benedetto, R., Maialetti, F., De Vincenzi, A., Calcaterra, R., Trecca, A., and De Vincenzi, M. (2008). A 1o-residue peptide from durum, wheat promotes a shift from a Th1-type response toward a Th2-type response in celiac disease. Am J Clin Nutr. 87:415-23.

Simpson, F.G., Howdle, P.D., Robertson, D.A., and Losowsky, M.S. (1983). Jejunal biopsy and lymphocyte co-culture in coeliac disease. Scand J Gastroenterol. 18:749-54.

Sjölander, A., and Magnusson, K.E. (1988). Effects of wheat germ agglutinin on the cellular content of filamentous actin in Intestine 407 cells. Eur J Cell Biol. 47:32-5.

Sjöström, H., Lundin, K.E., Molberg, O., Korner, R., McAdam, S.N., Anthonsen, D., Quarsten, H., Noren, O., Roepstorff, P., Thorsby, E., and Sollid, L.M. (1998). Identification of a gliadin T-cell epitope in coeliac disease: general importance of gliadin deamidation for intestinal T-cell recognition. Scand J Immunol. 48:111-5.

Smart, C.J., Trejdosiewicz, L.K., and Howdle, P.D. (1992). Specific circulating anti-gliadin IgG-class antibody does not mediate intestinal enteropathy in gliadin-fed mice. Int Arch Allergy Immunol. 97:160-6.

Smecuol, E., Bai, J.C., Vazquez, H., Kogan, Z., Cabanne, A., Niveloni, S., Pedreira, S., Boerr, L., Maurino, E., and Meddings, J.B. (1997). Gastrointestinal permeability in celiac disease. Gastroenterology. 112:1129-36.

Sollid, L.M., Markussen, G., Ek, J., Gjerde, H., Vartdal, F., and Thorsby, E. (1989). Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer. J Exp Med. 169:345-50.

Sollid, L.M., and Khosla, C. (2005). Future therapeutic options for celiac disease. Nat Clin Pract Gastroenterol Hepatol. 2:140-7.

Spaenij-Dekking, L., Kooy-Winkelaar, Y., van Veelen, P., Drijfhout, J.W., Jonker, H., van Soest, L., Smulders, M.J., Bosch, D., Gilissen, L.J., and Koning, F. (2005). Natural variation in toxicity of wheat: potential for selection of nontoxic varieties for celiac disease patients. Gastroenterology. 129:797-806.

Spencer, J., Isaacson, P.G., MacDonald, T.T., Thomas, A.J., and Walker-Smith, J.A. (1991). Gamma/delta T cells and the diagnosis of coeliac disease. Clin Exp Immunol. 85:109-13.

Stenson, W.F., Newberry, R., Lorenz, R., Baldus, C., and Civitelli, R. (2005). Increased prevalence of celiac disease and need for routine screening among patients with osteoporosis. Arch Intern Med. 165:393-9.

Stepniak, D., Spaenij-Dekking, L., Mitea, C., Moester, M., de Ru, A., Baak-Pablo, R., van Veelen, P., Edens, L., and Koning, F. (2006). Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. Am J Physiol Gastrointest Liver Physiol. 291:G621-9.

Stevenson, B.R., Siliciano, J.D., Mooseker, M.S., and Goodenough, D.A. (1986). Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol. 103:755-66.

Sturgess, R., Day, P., Ellis, H.J., Lundin, K.E., Gjertsen, H.A., Kontakou, M., and Ciclitira, P.J. (1994). Wheat peptide challenge in coeliac disease. Lancet. 343:758-61.

Sulkanen, S., Collin, P., Laurila, K., and Mäki, M. (1998a). IgA- and IgG-class antihuman umbilical cord antibody tests in adult coeliac disease. Scand J Gastroenterol. 33:251-4.

Sulkanen, S., Halttunen, T., Laurila, K., Kolho, K.L., Korponay-Szabo, I.R., Sarnesto, A., Savilahti, E., Collin, P., and Mäki, M. (1998b). Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. Gastroenterology. 115:1322-8.

Szakal, D.N., Gyorffy, H., Arato, A., Cseh, A., Molnar, K., Papp, M., Dezsofi, A., and Veres, G. (2010). Mucosal expression of claudins 2, 3 and 4 in proximal and distal part of duodenum in children with coeliac disease. Virchows Arch. 456:245-50.

Thompson, T., Dennis, M., Higgins, L.A., Lee, A.R., and Sharrett, M.K. (2005). Gluten-free diet survey: are Americans with coeliac disease consuming recommended amounts of fibre, iron, calcium and grain foods? J Hum Nutr Diet. 18:163-9.

Tiittanen, M., Westerholm-Ormio, M., Verkasalo, M., Savilahti, E., and Vaarala, O. (2008). Infiltration of forkhead box P3-expressing cells in small intestinal mucosa in coeliac disease but not in type 1 diabetes. Clin Exp Immunol. 152:498-507.

Tripathi, A., Lammers, K.M., Goldblum, S., Shea-Donohue, T., Netzel-Arnett, S., Buzza, M.S., Antalis, T.M., Vogel, S.N., Zhao, A., Yang, S., Arrietta, M.C., Meddings, J.B., and Fasano, A. (2009). Identification of human zonulin, a physiological modulator of tight junctions, as prehaptoglobin-2. Proc Natl Acad Sci U S A. 106:16799-804.

Turksen, K., and Troy, T.C. (2004). Barriers built on claudins. J Cell Sci. 117:2435-47.

Tuukkanen, K., Loponen, J., Mikola, M., Sontag-Strohm, T., and Salovaara, H. (2005). Degradation of secalins during rye sourdough fermentation. Cereal Chem. 82:677-682.

Tye-Din, J.A., Anderson, R.P., Ffrench, R.A., Brown, G.J., Hodsman, P., Siegel, M., Botwick, W., and Shreeniwas, R. (2009). The effects of ALV003 pre-digestion of gluten on immune response and symptoms in celiac disease in vivo. Clin Immunol.

Tye-Din, J.A., Stewart, J.A., Dromey, J.A., Beissbarth, T., van Heel, D.A., Tatham, A., Henderson, K., Mannering, S.I., Gianfrani, C., Jewell, D.P., Hill, A.V., McCluskey, J., Rossjohn, J., and Anderson, R.P. (2010). Comprehensive, quantitative mapping of T cell epitopes in gluten in celiac disease. Sci Transl Med. 2:41ra51.

Vader, L.W., de Ru, A., van der Wal, Y., Kooy, Y.M., Benckhuijsen, W., Mearin, M.L., Drijfhout, J.W., van Veelen, P., and Koning, F. (2002a). Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. J Exp Med. 195:643-9.

Vader, L.W., Stepniak, D.T., Bunnik, E.M., Kooy, Y.M., de Haan, W., Drijfhout, J.W., Van Veelen, P.A., and Koning, F. (2003). Characterization of cereal toxicity for celiac disease patients based on protein homology in grains. Gastroenterology. 125:1105-13.

Vader, W., Kooy, Y., Van Veelen, P., De Ru, A., Harris, D., Benckhuijsen, W., Pena, S., Mearin, L., Drijfhout, J.W., and Koning, F. (2002b). The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. Gastroenterology. 122:1729-37.

Wahab, P.J., Meijer, J.W., and Mulder, C.J. (2002). Histologic follow-up of people with celiac disease on a gluten-free diet: slow and incomplete recovery. Am J Clin Pathol. 118:459-63.

Wahnschaffe, U., Ullrich, R., Riecken, E.O., and Schulzke, J.D. (2001). Celiac disease-like abnormalities in a subgroup of patients with irritable bowel syndrome. Gastroenterology. 121:1329-38.

Waldmann, T.A., and Tagaya, Y. (1999). The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. Annu Rev Immunol. 17:19-49.

Walker, M.M., Murray, J.A., Ronkainen, J., Aro, P., Storskrubb, T., D'Amato, M., Lahr, B., Talley, N.J., and Agreus, L. (2010). Detection of Celiac Disease and Lymphocytic Enteropathy by Parallel Serology and Histopathology in a Population-Based Study. Gastroenterology.

Walker-Smith, J.A., Guandalini, S., Schmitz, J., Shmerling, D., and Visakorpi, J.K. (1990). Revised criteria for diagnosis of celiac disease. Arch Dis Child. 65:909-911.

Walz, F., Wieser, H., and Stern, M. (1996). In vitro hydrolysis of gliadin and casein peptides: secondary defect in coeliac disease shown by organ culture. Scand J Gastroenterol. 31:240-6.

Van De Kamer, J.H., Weijers, H.A., and Dicke, W.K. (1953). Coeliac disease. IV. An investigation into the injurious constituents of wheat in connection with their action on patients with coeliac disease. Acta Paediatr. 42:223-31.

van de Wal, Y., Kooy, Y.M., van Veelen, P.A., Pena, S.A., Mearin, L.M., Molberg, O., Lundin, K.E., Sollid, L.M., Mutis, T., Benckhuijsen, W.E., Drijfhout, J.W., and Koning, F. (1998). Small intestinal T cells of celiac disease patients recognize a natural pepsin fragment of gliadin. Proc Natl Acad Sci U S A. 95:10050-4.

van de Wal, Y., Kooy, Y.M., van Veelen, P., Vader, W., August, S.A., Drijfhout, J.W., Pena, S.A., and Koning, F. (1999). Glutenin is involved in the gluten-driven mucosal T cell response. Eur J Immunol. 29:3133-9.

van den Broeck, H.C., van Herpen, T., Schuit, C., Salentijn, E., Dekking, L., Bosch, D., Hamer, R.J., Smulders, M.J., Gilissen, L.J., and van den Meer, I.M. (2009). Removing celiac disease-related gluten proteins from wheat while retaining technological properties: a study with Chinese Spring deletion lines. BMC Plant Biol. 9:41.

van Heel, D.A., Franke, L., Hunt, K.A., Gwilliam, R., Zhernakova, A., Inouye, M., Wapenaar, M.C., Barnardo, M.C., Bethel, G., Holmes, G.K., Feighery, C., Jewell, D., Kelleher, D., Kumar, P., Travis, S., Walters, J.R., Sanders, D.S., Howdle, P., Swift, J., Playford, R.J., McLaren, W.M., Mearin, M.L., Mulder, C.J., McManus, R., McGinnis, R., Cardon, L.R., Deloukas, P., and Wijmenga, C. (2007). A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. Nat Genet. 39:827-9.

Verdu, E.F., Huang, X., Natividad, J., Lu, J., Blennerhassett, P.A., David, C.S., McKay, D.M., and Murray, J.A. (2008). Gliadin-dependent neuromuscular and epithelial secretory responses in gluten-sensitive HLA-DQ8 transgenic mice. Am J Physiol Gastrointest Liver Physiol. 294:G217-25.

Wieser, H. (1994). Quantitative determination of gliadin subgroups from different wheat cultivars. J Cereal Science. 19:149-155.

Viljamaa, M., Kaukinen, K., Pukkala, E., Hervonen, K., Reunala, T., and Collin, P. (2006). Malignancies and mortality in patients with coeliac disease and dermatitis herpetiformis: 30-year population-based study. Dig Liver Dis. 38:374-80.

Vilppula, A., Collin, P., Mäki, M., Valve, R., Luostarinen, M., Krekela, I., Patrikainen, H., Kaukinen, K., and Luostarinen, L. (2008). Undetected coeliac disease in the elderly: a biopsy-proven population-based study. Dig Liver Dis. 40:809-13.

Vilppula, A., Kaukinen, K., Luostarinen, L., Krekela, I., Patrikainen, H., Valve, R., Mäki, M., and Collin, P. (2009). Increasing prevalence and high incidence of celiac disease in elderly people: a population-based study. BMC Gastroenterol. 9:49.

Vogelsang, H., Schwarzenhofer, M., Granditsch, G., and Oberhuber, G. (1999). In vitro production of endomysial antibodies in cultured duodenal mucosa from patients with celiac disease. Am J Gastroenterol. 94:1057-61.

Volta, U., De Franceschi, L., Lari, F., Molinaro, N., Zoli, M., and Bianchi, F.B. (1998). Coeliac disease hidden by cryptogenic hypertransaminasaemia. Lancet. 352:26-9.

Xia, J., Bergseng, E., Fleckenstein, B., Siegel, M., Kim, C.Y., Khosla, C., and Sollid, L.M. (2007). Cyclic and dimeric gluten peptide analogues inhibiting DQ2-mediated antigen presentation in celiac disease. Bioorg Med Chem. 15:6565-73.

Yokoyama, S., Watanabe, N., Sato, N., Perera, P.Y., Filkoski, L., Tanaka, T., Miyasaka, M., Waldmann, T.A., Hiroi, T., and Perera, L.P. (2009). Antibody-mediated blockade of IL-15 reverses the autoimmune intestinal damage in transgenic mice that overexpress IL-15 in enterocytes. Proc Natl Acad Sci U S A. 106:15849-54.

Young, W.F., and Pringle, E.M. (1971). 110 children with coeliac disease, 1950-1969. Arch Dis Child. 46:421-36.

Zanoni, G., Navone, R., Lunardi, C., Tridente, G., Bason, C., Sivori, S., Beri, R., Dolcino, M., Valletta, E., Corrocher, R., and Puccetti, A. (2006). In celiac disease, a subset of autoantibodies against transglutaminase binds toll-like receptor 4 and induces activation of monocytes. PLoS Med. 3:e358.

Zhong, F., McCombs, C.C., Olson, J.M., Elston, R.C., Stevens, F.M., McCarthy, C.F., and Michalski, J.P. (1996). An autosomal screen for genes that predispose to celiac disease in the western counties of Ireland. Nat Genet. 14:329-33.

Zimmer, K.P., Poremba, C., Weber, P., Ciclitira, P.J., and Harms, E. (1995). Translocation of gliadin into HLA-DR antigen containing lysosomes in coeliac disease enterocytes. Gut. 36:703-9.

Zimmer, K.P., Fischer, I., Mothes, T., Weissen-Plenz, G., Schmitz, M., Wieser, H., Mendez, E., Buening, J., Lerch, M.M., Ciclitira, P.C., Weber, P., and Naim, H.Y. (2009). Endocytotic Segregation of Gliadin Peptide 31-49 in Enterocytes. Gut.

BMC Immunology

Research article

Secretion of celiac disease autoantibodies after in vitro gliadin challenge is dependent on small-bowel mucosal transglutaminase 2-specific IgA deposits

Satumarja M Stenman[1], Katri Lindfors[1], Ilma R Korponay-Szabo[1,2], Olli Lohi[1,6], Päivi Saavalainen[3], Jukka Partanen[4], Katri Haimila[4], Herbert Wieser[5], Markku Mäki[1,6] and Katri Kaukinen*[1,7]

Address: [1]Medical School, Pediatric Research Center, University of Tampere, Tampere, Finland, [2]Heim Pal Childrens' Hospital, Budapest, Hungary, [3]Department of Medical Genetics, University of Helsinki, Helsinki, Finland, [4]Finnish Red Cross Blood Service, Helsinki, Finland, [5]Deutsche Forschungsanstalt für Lebensmittelchemie, Garching, Germany, [6]Department of Pediatrics, Tampere University Hospital, Tampere, Finland and [7]Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital, Tampere, Finland

Email: Satumarja M Stenman - satumarja.stenman@uta.fi; Katri Lindfors - katri.lindfors@uta.fi; Ilma R Korponay-Szabo - Ilma.Korponay-Szabo@uta.fi; Olli Lohi - olli.lohi@uta.fi; Päivi Saavalainen - paivi.saavalainen@helsinki.fi; Jukka Partanen - jukka.partanen@veripalvelu.fi; Katri Haimila - katri.haimila@veripalvelu.fi; Herbert Wieser - H.Wieser@lrz.tu-muenchen.de; Markku Mäki - markku.maki@uta.fi; Katri Kaukinen* - katri.kaukinen@uta.fi

* Corresponding author

Published: 29 February 2008

BMC Immunology 2008, **9**:6 doi:10.1186/1471-2172-9-6

Received: 30 November 2007
Accepted: 29 February 2008

This article is available from: http://www.biomedcentral.com/1471-2172/9/6

Abstract

Background: In celiac disease gluten, the disease-inducing toxic component in wheat, induces the secretion of autoantibodies which are targeted against transglutaminase 2 (TG2). These autoantibodies are produced in the small-intestinal mucosa, where they can be found deposited extracellularly below the epithelial basement membrane and around mucosal blood vessels. In addition, during gluten consumption these autoantibodies can also be detected in patients' serum but disappear from the circulation on a gluten-free diet. Interestingly, after adoption of a gluten-free diet the serum autoantibodies disappear from the circulation more rapidly than the small-intestinal mucosal autoantibody deposits. The toxicity of gluten and the secretion of the disease-specific autoantibodies have been widely studied in organ culture of small-intestinal biopsy samples, but results hitherto have been contradictory. Since the mucosal autoantibodies disappear slowly after a gluten-free diet, our aim was to establish whether autoantibody secretion to organ culture supernatants in treated celiac disease patient biopsies is related to the duration of the diet and further to the pre-existence of mucosal TG2-specific IgA deposits in the cultured biopsy samples.

Results: In the organ culture system conducted with biopsies derived from treated celiac disease patients, gliadin induced secretion of autoantibodies to culture supernatants, reduced epithelial cell height and increased the density of *lamina propria* CD25+ cells. However, these changes could be demonstrated only in biopsies from short-term treated celiac disease patients, where the small-intestinal mucosal TG2-specific IgA autoantibody deposits were still present. Furthermore, in these biopsies autoantibody secretion could be stimulated fully only after a 48-hour gliadin challenge.

Conclusion: Our results show that studies focusing on the toxic effects of gliadin in the organ culture system should be carried out with biopsy samples from short-term treated celiac disease patients who are likely still to have mucosal IgA deposits present. In addition to providing an explanation for the discrepancies in previous publications, the present study also enables further validation of the organ culture method.

Background

Celiac disease is a gluten-induced autoimmune disease of the small intestine characterized by small-bowel mucosal villous atrophy with crypt hyperplasia and a profound inflammation in the mucosa. In addition to causing damage to the mucosa in genetically susceptible individuals, gluten also provokes the production of autoantibodies typically found in the sera of untreated celiac disease patients. These autoantibodies recognize exclusively endomysial antigens now identified as transglutaminase 2 (TG2). The autoantibodies are produced locally in the mucosa [1,2], and besides being detectable in patient sera, they are also deposited extracellularly *in vivo* in the mucosa [3-7]. Furthermore, recent findings suggest that these TG2-targeted mucosal IgA-autoantibody deposits are already present in the early phases of the disease process prior to manifest mucosal lesion [4,5] and before autoantibodies appear in the serum [3-6]. After adoption of a gluten-free diet, serum autoantibodies disappear and the small-bowel mucosa heals usually within one year [8]. At the time the autoantibodies have disappeared from the circulation, there may still be residual autoantibody deposits present in the small-intestinal mucosa which will also in due course disappear on a strict gluten-free diet [4,6].

For over twenty years the human small-intestinal organ culture method has been widely used in detecting the toxic effects of wheat gliadin in celiac disease *in vitro* [1,9-15]. In earlier studies the toxicity of gliadin has commonly been demonstrated by an increased density of *lamina propria* lymphocytes [11-13] and reduced epithelial cell height (ECH) [14,16] in cultured biopsy samples from untreated and treated celiac disease patients. However, when measuring endomysial autoantibody (EmA) secretion to culture supernatants from biopsies from treated celiac patients, the results have been contradictory. Picarelli and associates [1] showed that in biopsies derived from treated celiac disease patients gliadin induces secretion of EmA to culture supernatants. In contrast, some studies report that the secretion of EmA can only be achieved in half of [14,17] or even no [18] experiments carried out with treated celiac patient biopsies. And even further, it has been suggested that EmA secretion to the organ culture system is totally independent of gliadin challenge [18] and histological damage [14].

Due to these discrepancies among previous studies concerning autoantibody secretion to the organ culture system, our aim was to establish in both short- and long-term treated celiac disease patients whether the antibody secretion to culture supernatants is dependent on the duration of patients' gluten-free diet (GFD). Furthermore, since in celiac disease the small-bowel mucosal extracellular TG2-specific IgA deposits seem to disappear slowly after a gluten-free diet [6], we hypothesized that the autoantibody secretion to supernatants is related to the existence of mucosal TG2-specific IgA deposits in the cultured small-bowel biopsy samples.

Results

Celiac autoantibodies

In order to study the baseline serum autoantibody levels of study subjects, EmA and TG2 antibody (TG2-ab) titers were measured. All five untreated celiac disease patients involved had positive EmA (median titer 1:500, range 5–4000) and TG2-ab titers (median titer 58.1, range 4.7–100) in serum. In contrast, all 20 treated celiac disease patients and all six non-celiac controls had normal serum autoantibody levels. Furthermore, all celiac disease patients carried either the HLA DQ2 or the DQ8 haplotype. When the small-bowel biopsies from celiac disease patients were challenged in the organ culture system with peptic-tryptic digest of gliadin (PT-gliadin) for 48 hours, EmA was secreted to the culture supernatants in all five untreated but in only 11 out of the 20 treated celiac cases (Table 1). Moreover, no antibodies were found in supernatants from non-celiac controls (Table 1).

When autoantibody secretion to culture supernatants was investigated in relation to the duration of the gluten-free diet, EmA was detected in supernatants after PT-gliadin challenge in 10 out of 12 short-term (GFD 1–3 years) and in 1 out of 8 long-term (GFD 4–20 years) treated patient biopsy samples (Table 2, Table 3). Since the duration of the gluten-free diet was insufficient to explain EmA secretion completely, we tested whether EmA presence in the culture supernatants was dependent on pre-existing small-

Table 1: Endomysial antibodies (EmA) in organ culture supernatants after 48 hours' *in vitro* PT-gliadin challenge in untreated and treated celiac disease patients and non-celiac controls.

	EmA after 48 h *in vitro* PT-gliadin challenge	
	Positive	**Negative**
Untreated celiac disease (n = 5)	100%	
Treated celiac disease (n = 20)	55%	45%
Non-celiac control (n = 6)		100%

Table 2: Antiendomysial (EmA) and transglutaminase-2 antibody (TG2-ab) secretion to organ culture supernatants after *in vitro* PT-gliadin challenge. Organ culture of treated celiac disease patient biopsy samples (n = 11) who had small-bowel mucosal IgA deposits. Small-intestinal biopsy samples were cultured either with medium only or with PT-gliadin for 24 or 48 hours. EmA titers were graded according to intensity of staining as follows: negative (neg), weak positive (+) and strong positive (++, +++ or ++++).

		EmA				TG2-ab (U)		
Patient	**GFD years**	**Medium 24 h**	**PT-gliadin24 h**	**PT-gliadin 48 h**		**Medium 24 h**	**PT-gliadin 24 h**	**PT-gliadin 48 h**
1	1	++	+++	++++		11.0	17.1	37.8
2	1	++	++	+++		7.3	11.8	30.3
3	1	++	++	+++		16.0	6.3	26.6
4	1	+	++	+++		12.4	13.4	9.5
5	1	++	neg	++		17.2	7.4	22.9
6	1	neg	++	++		6.5	13.1	19.9
7	1	neg	neg	+		7.6	5.9	24.5
8	2	neg	+	++		7.8	5.3	14.8
9	3	+	++	++++		21.0	10.3	30.9
10	3	neg	++	++		4.9	7.0	nd
11	4	++	++	nd		24.5	11.9	nd
Median		**+**	++	**+++***	**Median**	**11.0**	**10.3**	**24.5†**

nd = not determined
GFD = gluten-free diet
* P < 0.01; † P = 0.011 compared to medium only 24 hours and PT-gliadin 24 hours.

Table 3: Antiendomysial (EmA) and transglutaminase-2 antibody (TG2-ab) secretion to organ culture supernatants after *in vitro* PT-gliadin challenge. Organ culture of treated celiac disease patient biopsy samples (n = 9) who had no small-bowel mucosal IgA deposits. Small-intestinal biopsy samples were cultured either with medium only or with PT-gliadin for 24 or 48 hours. EmA titers were graded according to intensity of staining as follows: negative (neg), weak positive (+) and strong positive (++, +++ or ++++).

		EmA				TG2-ab (U)		
Patient	**GFD years**	**Medium 24 h**	**PT-gliadin 24 h**	**PT-gliadin 48 h**		**Medium 24 h**	**PT-gliadin 24 h**	**PT-gliadin 48 h**
12	1	neg	neg	neg		2.0	4.8	13.3
13	2	neg	neg	neg		8.8	6.9	4.4
14	4	neg	neg	neg		2.2	6.2	10.3
15	4	neg	neg	neg		2.5	1.7	2.6
16	6	neg	neg	neg		8.1	7.7	12.4
17	7	neg	neg	neg		2.4	3.7	1.9
18	8	neg	neg	neg		2.5	1.3	1.7
19	14	neg	neg	neg		0.9	2.9	0.7
20	20	neg	neg	neg		11.6	2.5	11.6
Median		**neg**	**neg**	**neg**	**Median**	**2.5**	**3.7**	**4.4***

GFD = gluten-free diet
* P > 0.05 compared to medium only 24 hours and PT-gliadin 24 hours

bowel mucosal IgA-autoantibody deposits. When the treated celiac patients were divided into two groups based on the presence or absence of mucosal IgA autoantibody deposits (Figure 1), it was found that only biopsies derived from patients having positive autoantibody deposits were able to secrete EmA to the culture supernatants (Table 2, Table 3). In some treated celiac cases evincing mucosal IgA deposits, EmA was already secreted to the supernatant spontaneously without PT-gliadin stimulation, but both EmA and TG2 antibody titers increased significantly after 48 hours' culture with PT-gliadin (Table 2). Although the autoantibody titers increased in culture supernatants after PT-gliadin challenge, there was no significant change in the intensity of mucosal IgA deposits in

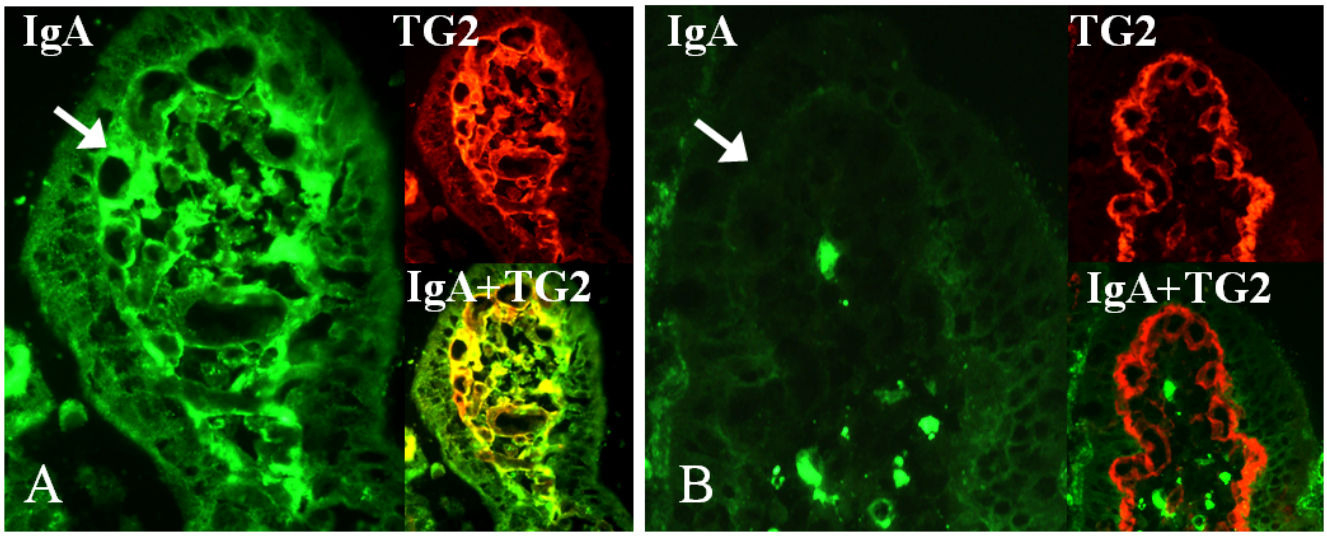

Figure 1
Small-bowel mucosal transglutaminase 2 (TG2, red)-specific IgA deposits (green) after 24 hours' organ culture. A) Positive staining (arrow) in the mucosal villous of a short-term treated celiac disease patient (gluten-free diet for three years). B) Negative IgA deposits (arrow) in the small-bowel mucosa of long-term treated celiac disease patient (gluten-free diet for eight years). Co-localization of IgA deposits with TG2 is shown in yellow. Magnification 40×.

the cultured biopsy samples after the challenge (data not shown).

Determination of ECH and the number of CD25+ lymphocytes

In further demonstrating the toxic effects of gliadin, ECH and the number of *lamina proprial* CD25+ cells were calculated in cultured biopsy samples. In summary, it was found that these parameters were also dependent on the presence of mucosal IgA deposits. The ECH decreased and the density of CD25+ cells increased significantly only in the treated celiac disease patient biopsies with pre-existing IgA deposits (Figure 2 and 3).

Discussion

The results presented here show that gliadin toxicity, as measured by autoantibody secretion, decrease in ECH and increase in the density of *lamina proprial* CD25+ cells, can be demonstrated in the treated celiac disease patient organ culture system only if small-intestinal mucosal TG2-specific IgA autoantibody deposits are still present in the cultured biopsy samples. Patients with persisting mucosal autoantibody deposits, normal small-bowel mucosal villous structure and negative serum autoantibody levels are usually those who have been on a gluten-free diet for a short period of time.

Our results explain the discrepancies reported in previous papers on EmA secretion in biopsies derived from treated celiac disease patients. Vogelsang and colleagues [18]

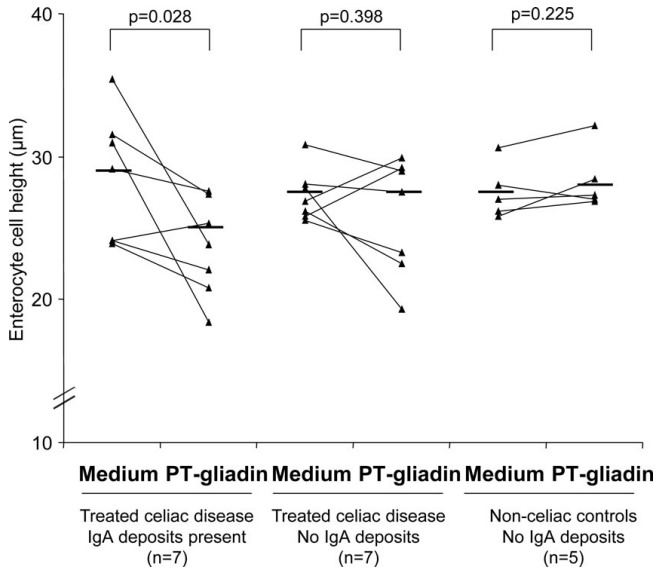

Figure 2
Enterocyte cell height (ECH) after organ culture. Biopsy samples of treated celiac disease patients with and without mucosal IgA deposits and non-celiac control patients. Biopsies were cultured with medium only or with PT-gliadin for 24 hours. The median values (horizontal line) and P values are calculated for each group showing a statistically significant decrease in ECH only in those treated celiac disease cases who had small-bowel mucosal IgA deposits in the cultured biopsy samples.

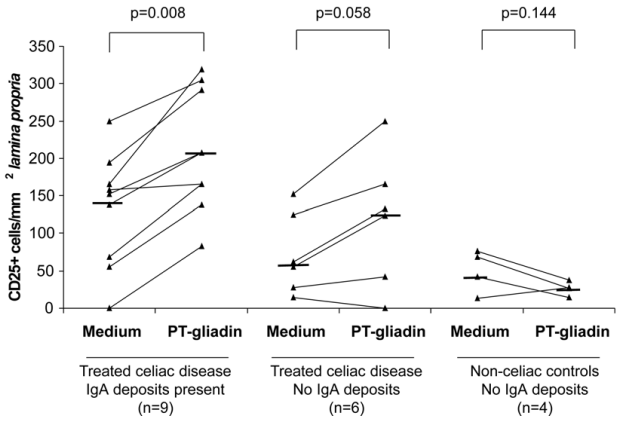

Figure 3

The total number of mucosal *lamina propria* CD25-positive T-cells. Cultured biopsy samples from treated celiac disease patients with and without mucosal IgA deposits and non-celiac control patients. Biopsies were cultured either with medium only or with PT-gliadin for 24 hours. The median values (horizontal line) and P values are calculated for each group showing a statistically significant increase in CD25+ cells only in those treated celiac disease cases who had small-bowel mucosal IgA deposits in the cultured biopsy samples.

reported that EmA secretion after a 24-hour gliadin challenge was achieved in only 2 out of 18 (11%) biopsy samples derived from celiac disease patients on a strict gluten-free diet with normal mucosal villous morphology. In contrast, biopsies from 60% of patients with dietary lapses and 92% of untreated celiacs with ongoing mucosal inflammation responded to the challenge in respect of EmA secretion. Similar results have been obtained by Biagi and associates [14] in a small-scale study involving only seven treated celiac disease patients. EmA secretion to the culture supernatant could not be stimulated after a 24-hour gliadin administration in any of the long-term (8–30 years) treated celiac patients with normal mucosal architecture. Samples responding to the gliadin insult were derived from patients still evincing mucosal damage despite a gluten-free diet. In the above-mentioned studies the treated patients unable to react to gliadin as measured by EmA secretion were well and long-term treated and may be hypothesized not to have small-bowel IgA autoantibody deposits. This absence of mucosal deposits could thus explain the discrepancy in results. Furthermore, it has been shown that 24 hours of gliadin treatment might not be a sufficient time to induce EmA secretion, as shown by Picarelli and colleagues [19] who demonstrated that a 48-hour culture period in the presence of gliadin is needed to achieve EmA secretion in all treated celiac disease patient samples, this also being in agreement with the results presented in the current study (Table 2).

It remains to be elucidated how a gliadin challenge leads to EmA secretion in the organ culture system. Basically there are two different possibilities. Firstly, EmA could be actively secreted by plasma cells in the biopsy specimen [1]. The inability of long-term treated patient biopsy samples to secrete autoantibodies to the supernatant could be explained by the overall absence of plasma cells and helper T cells as well as the failure of memory B cells to become activated within the timeframe of the organ culture [17,18]. Secondly, the secretion of EmA to the culture supernatant might simply be due to detachment of the autoantibodies from the tissue deposits and their release into the supernatant. However, we observed no decrease in the intensity of the mucosal autoantibody deposits after the organ culture. We did nevertheless see changes in ECH and the density of CD25+ cells, which speaks in favor of an active gliadin-induced process, this supporting the first proposition.

Conclusion

Our results indicate that when studying the toxic effects of gliadin in an organ culture setting, biopsy samples from short-term treated celiac disease patients who are likely still to have mucosal TG2-specific IgA deposits should be used. Altogether, the current study provides a platform for further validation of the organ culture method.

Methods

Patients

Small-bowel mucosal biopsies were taken from 20 treated patients suffering from celiac disease (median age 54 years, range 23–73 years, females 80%), five untreated celiac patients (median age 48 years, range 43–71 years, females 100%) and six non-celiac control subjects suffering from dyspepsia (median age 53 years, range 24–70 years, females 50%). In all celiac patients the diagnosis was initially based on the European Society of Pediatric Gastroenterology and Nutrition criteria [20], meaning that they all had small-bowel mucosal villous atrophy with crypt hyperplasia in the duodenum while consuming gluten. All treated celiac patients involved in the current study had been on a strict gluten-free diet for at least one year (median duration of GFD three years, range 1–20 years), and all, as well as the non-celiac controls, showed normal small-bowel mucosal architecture. In contrast, all the untreated patients evinced subtotal villous atrophy with crypt hyperplasia in the small-bowel mucosa. The study protocol was accepted by the Ethical Committee of Tampere University Hospital and written informed consent was obtained from all patients and controls.

Small-bowel mucosal biopsies and organ culture system

Altogether seven small-bowel mucosal biopsy samples were obtained from each patient during upper gastrointestinal endoscopy. Two samples were immediately snap-frozen in liquid nitrogen with optimal cutting temperature compound (OCT, Tissue-Tek, Sakura Finetek Europe, Holland) and stored at -20°C until used. Further, two biopsies were immediately fixed in paraffin for investigation of the baseline small-bowel mucosal morphology. The remaining three biopsies were cultured for 24 or 48 hours at 37°C, either in the presence or absence of a peptic-tryptic digest of gliadin (1 mg/ml) prepared by a standard protocol described elsewhere [15,21].

The organ culture method was implemented as originally described by Browning and Trier [9]. Briefly, the biopsy samples were cultured in RPMI-1640 medium (Invitrogen-Gibco, Paisley, Scotland, UK) containing 15 % heat-inactivated fetal bovine serum (Invitrogen-Gibco), 100 μg/ml streptomycin (Invitrogen-Gibco), 100 U/ml penicillin (Invitrogen-Gibco), 4 mM L-glutamine (Invitrogen-Gibco), 50 μg/ml insulin (Sigma-Aldrich Co, St. Louis, Missouri, USA) and 10 mM HEPES buffer (Invitrogen-Gibco). Duodenal specimens were placed villi upwards on a sterile stainless-steel grid positioned over the medium in a central well of the organ culture dish (Falcon, Becton Dickinson and Co, USA). After 24 or 48 hours' incubation, culture supernatants were collected and stored at -70°C until analysed. Free fluid was removed from the samples, whereafter they were snap-frozen with OCT and stored at -20°C until processed for stainings.

Celiac autoantibodies

EmA was detected in patients' serum and undiluted organ culture supernatants using an indirect immunofluorescence assay where human umbilical cord was used as antigen [8]. A serum dilution of 1:≥5 was considered positive. Antibody titers for organ culture supernatants were graded according to the intensity of the staining as follows: negative (neg), weak positive (+) and strong positive (++, +++ or ++++). Samples were analyzed blindly without knowledge of the patients' clinical background.

TG2-antibodies were measured by enzyme-linked immunosorbent assay (ELISA, Celikey®, Phadia, Freiburg, Germany), according to manufacturer's instructions, both in serum samples (diluted 1:100) and in undiluted culture supernatants. In serum samples a unit value (U) ≥ 5U was considered positive. Since there is no cut-off value for TG2-antibody in culture supernatants, the crude antibody values are given only for comparison to EmA.

Small-bowel mucosal TG2-specific IgA deposits

The small-bowel mucosal TG2-targeted IgA deposits were investigated before and after 24 hours of organ culture. In earlier studies it has been shown that these mucosal IgA deposits are specifically targeted against TG2 in the small-bowel mucosa [3,6]. In the studies in question, autoantibody specificity for TG2 was demonstrated by the fact that IgA eluted from duodenal mucosa bound intensively to purified TG2 in ELISA and Western blot [3]. Similarly, a human recombinant TG2 was capable of depositing celiac-specific IgA in small-bowel sections from celiac disease patients [6]. In addition, after removal of TG2 from the sections by a specific acid, both TG2 and IgA deposits disappeared from the mucosa [6].

To study the mucosal IgA deposits the 5-μm-thick unfixed cryostat sections were stained using a two-color immunofluorescence method as previously described [3]. The monoclonal primary antibody against TG2 (Clone CUB 7402, NeoMarkers, Fremont, USA, 1:200) was used followed by the rhodamine-conjugated antimouse immunoglobulin antibody (Dako, A/S, Glostrup, Denmark 1:120) and the fluorescein isothiocyanate-conjugated rabbit antibody against human IgA (Dako, 1:40). In untreated celiac disease a clear extracellular subepithelial IgA deposition can be found below the basement membrane along the villous and crypt epithelium and around mucosal vessels; this is in contrast to non-celiac normal small-bowel samples, where IgA is detected only inside plasma and epithelial cells [4,6,22].

Determination of ECH and the number of CD25+ lymphocytes

ECH was measured under a light microscope (Olympus BX60, 40× magnification) after 24 hours' organ culture with or without PT-gliadin challenge using the analySIS 3.0 program, (Soft Imaging System GmbH, Munster, Germany). Altogether 30 enterocytes from three different villi of each specimen were analyzed and a mean ECH value was calculated for each biopsy sample [14].

CD25-positive T cells were detected in the *lamina propria* of small-bowel mucosa from biopsy samples cultured for 24 hours with or without PT-gliadin. The 5-μm-thick cryostat sections were fixed in acetone and incubated with goat normal serum (Vector Laboratories Inc., Burlingame, USA), whereafter they were incubated with mouse monoclonal antibody, human anti-CD25 (Dako, 1:25) for one hour and alexa-conjugated goat anti-mouse IgG (Invitrogen, 1:1000) for 30 minutes. Washes with PBS were performed between each antibody. The density of small-bowel mucosal CD25-positive T-cells in the *lamina propria* was calculated and presented as number of cells in a total area of one mm^2 of *lamina propria* [12,13].

HLA-typing

Celiac disease is strongly associated with the HLA gene region, since over 95% of celiac disease patients have either HLA DQ2 or HLA DQ8 haplotype molecules [23,24]. HLA-DQ typing was performed in each patient using DELFIA® Celiac Disease Hybridization Assay (PerkinElmer Life and Analytic Sciences, Wallac Oy, Turku, Finland).

Statistical analysis

Statistical analysis was performed using 2-tailed Wilcoxon Signed Ranks Test or Mann-Whitney U Test, as appropriate. P values lower than 0.05 were considered statistically significant.

Authors' contributions

SS designed the study, carried out the experiments, collected data and drafted the manuscript

KL designed the study, supervised the work, participated in the writing of the manuscript and provided funding

IKS designed the study, analyzed the data and revised the manuscript

OL designed the study and revised the manuscript

PS performed the HLA-typing of patients and revised the manuscript

JP performed the HLA-typing of patients and revised the manuscript

KH performed the HLA-typing of patients and revised the manuscript

HW prepared the PT-gliadin used in the experiments and revised the manuscript

MM originated the idea for the research, provided funding and revised the manuscript

KK supervised the work, studied the patients and participated in the writing of the manuscript

All authors have read and approved the final manuscript.

Acknowledgements

HW thanks U. Schuetzler for excellent technical assistance. The present study and the Celiac Disease Study Group are supported by grants from the Competitive Research Funding of the Pirkanmaa Hospital District, the Yrjö Jahnsson Foundation, the Finnish Medical Foundation, the Research Fund of the Finnish Celiac Society and the Academy of Finland Research Council for Health.

References

1. Picarelli A, Maiuri L, Frate A, Greco M, Auricchio S, Londei M: **Production of antiendomysial antibodies after in-vitro gliadin challenge of small intestine biopsy samples from patients with coeliac disease.** *Lancet* 1996, **348:**1065-1067.
2. Marzari R, Sblattero D, Florian F, Tongiorgi E, Not T, Tommasini A, Ventura A, Bradbury A: **Molecular dissection of the tissue transglutaminase autoantibody response in celiac disease.** *J Immunol* 2001, **166:**4170-4176.
3. Korponay-Szabo IR, Halttunen T, Szalai Z, Laurila K, Kiraly R, Kovacs JB, Fesus L, Maki M: **In vivo targeting of intestinal and extraintestinal transglutaminase 2 by coeliac autoantibodies.** *Gut* 2004, **53:**641-648.
4. Kaukinen K, Peraaho M, Collin P, Partanen J, Woolley N, Kaartinen T, Nuutinen T, Halttunen T, Maki M, Korponay-Szabo I: **Small-bowel mucosal transglutaminase 2-specific IgA deposits in coeliac disease without villous atrophy: A prospective and randomized clinical study.** *Scand J Gastroenterol* 2005, **40:**564-572.
5. Salmi TT, Collin P, Jarvinen O, Haimila K, Partanen J, Laurila K, Korponay-Szabo IR, Huhtala H, Reunala T, Maki M, Kaukinen K: **Immunoglobulin A autoantibodies against transglutaminase 2 in the small intestinal mucosa predict forthcoming coeliac disease.** *Aliment Pharmacol Ther* 2006, **24:**541-552.
6. Salmi TT, Collin P, Korponay-Szabo IR, Laurila K, Partanen J, Huhtala H, Kiraly R, Lorand L, Reunala T, Maki M, Kaukinen K: **Endomysial antibody-negative coeliac disease: clinical characteristics and intestinal autoantibody deposits.** *Gut* 2006.
7. Hadjivassiliou M, Maki M, Sanders DS, Williamson CA, Grunewald RA, Woodroofe NM, Korponay-Szabo IR: **Autoantibody targeting of brain and intestinal transglutaminase in gluten ataxia.** *Neurology* 2006, **66:**373-377.
8. Sulkanen S, Halttunen T, Laurila K, Kolho KL, Korponay-Szabo IR, Sarnesto A, Savilahti E, Collin P, Maki M: **Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease.** *Gastroenterology* 1998, **115:**1322-1328.
9. Browning TH, Trier JS: **Organ culture of mucosal biopsies of human small intestine.** *J Clin Invest* 1969, **48:**1423-1432.
10. Jos J, Lenoir G, Ritis GD, Rey J: **In vitro pathogenetic studies of coeliac disease. Effects of protein digests on coeliac intestinal biopsy specimens maintained in culture for 48 hours.** *Scand J Gastroenterol* 1975, **10:**121-128.
11. Maiuri L, Picarelli A, Boirivant M, Coletta S, Mazzilli MC, De Vincenzi M, Londei M, Auricchio S: **Definition of the initial immunologic modifications upon in vitro gliadin challenge in the small intestine of celiac patients.** *Gastroenterology* 1996, **110:**1368-1378.
12. Maiuri L, Ciacci C, Ricciardelli I, Vacca L, Raia V, Auricchio S, Picard J, Osman M, Quaratino S, Londei M: **Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease.** *Lancet* 2003, **362:**30-37.
13. Salvati VM, Mazzarella G, Gianfrani C, Levings MK, Stefanile R, De Giulio B, Iaquinto G, Giardullo N, Auricchio S, Roncarolo MG, Troncone R: **Recombinant human interleukin 10 suppresses gliadin dependent T cell activation in ex vivo cultured coeliac intestinal mucosa.** *Gut* 2005, **54:**46-53.
14. Biagi F, Parnell ND, Ellis HJ, Ciclitira PJ: **Endomysial antibody production is not related to histological damage after in vitro gluten challenge.** *Eur J Gastroenterol Hepatol* 2000, **12:**57-60.
15. Kilmartin C, Lynch S, Abuzakouk M, Wieser H, Feighery C: **Avenin fails to induce a Th1 response in coeliac tissue following in vitro culture.** *Gut* 2003, **52:**47-52.
16. Shidrawi RG, Day P, Przemioslo R, Ellis HJ, Nelufer JM, Ciclitira PJ: **In vitro toxicity of gluten peptides in coeliac disease assessed by organ culture.** *Scand J Gastroenterol* 1995, **30:**758-763.
17. Carroccio A, Iacono G, D'Amico D, Cavataio F, Teresi S, Caruso C, Di PL, Colombo A, D'Arpa F, Florena A, Notarbartolo A, Montalto G: **Production of anti-endomysial antibodies in cultured duodenal mucosa: usefulness in coeliac disease diagnosis.** *Scand J Gastroenterol* 2002, **37:**32-38.
18. Vogelsang H, Schwarzenhofer M, Granditsch G, Oberhuber G: **In vitro production of endomysial antibodies in cultured duodenal mucosa from patients with celiac disease.** *Am J Gastroenterol* 1999, **94:**1057-1061.
19. Picarelli A, Sabbatella L, Di Tola M, Vetrano S, Maffia C, Picchi C, Mastracchio A, Paoluzi P, Anania MC: **Forty-eight hours of biopsy cul-**

ture improve the sensitivity of the in vitro gliadin challenge in the diagnosis of celiac disease. *Clin Chem* 2001, **47:**1841-1843.
20. **Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition.** *Arch Dis Child* 1990, **65:**909-911.
21. Bolte G, Osman A, Mothes T, Stern M: **Peptic-tryptic digests of gliadin: contaminating trypsin but not pepsin interferes with gastrointestinal protein binding characteristics.** *Clin Chim Acta* 1996, **247:**59-70.
22. Korponay-Szabo IR, Laurila K, Szondy Z, Halttunen T, Szalai Z, Dahlbom I, Rantala I, Kovacs JB, Fesus L, Maki M: **Missing endomysial and reticulin binding of coeliac antibodies in transglutaminase 2 knockout tissues.** *Gut* 2003, **52:**199-204.
23. Polvi A, Arranz E, Fernandez-Arquero M, Collin P, Maki M, Sanz A, Calvo C, Maluenda C, Westman P, de la Concha EG, Partanen J: **HLA-DQ2-negative celiac disease in Finland and Spain.** *Hum Immunol* 1998, **59:**169-175.
24. Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F, Thorsby E: **Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer.** *J Exp Med* 1989, **169:**345-350.

Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2010.04119.x

Degradation of coeliac disease-inducing rye secalin by germinating cereal enzymes: diminishing toxic effects in intestinal epithelial cells

S. M. Stenman,* K. Lindfors,* J. I. Venäläinen,† A. Hautala,* P. T. Männistö,‡ J. A. Garcia-Horsman,‡ A. Kaukovirta-Norja,§ S. Auriola,¶ T. Mauriala,** M. Mäki*†† and K. Kaukinen*‡‡

*Pediatric Research Center, Medical School, University of Tampere, ††Department of Pediatrics, ‡‡Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital, Tampere, †Department of Pharmacology and Toxicology, ¶Department of Pharmaceutical Chemistry, University of Kuopio, Kuopio, ‡Division of Pharmacology and Toxicology, **Division of Pharmaceutical Chemistry, University of Helsinki, Helsinki, and §Technical Research Centre of Finland, Espoo, Finland

Accepted for publication 18 January 2010
Correspondence: K. Kaukinen, Paediatric Research Centre, Medical School, Finn-Medi 3, FI-33014 University of Tampere, Finland.
E-mail: katri.kaukinen@uta.fi

Summary

Currently the only treatment for coeliac disease is a lifelong gluten-free diet excluding food products containing wheat, rye and barley. There is, however, only scarce evidence as to harmful effects of rye in coeliac disease. To confirm the assumption that rye should be excluded from the coeliac patient's diet, we now sought to establish whether rye secalin activates toxic reactions *in vitro* in intestinal epithelial cell models as extensively as wheat gliadin. Further, we investigated the efficacy of germinating cereal enzymes from oat, wheat and barley to hydrolyse secalin into short fragments and whether secalin-induced harmful effects can be reduced by such pretreatment. In the current study, secalin elicited toxic reactions in intestinal Caco-2 epithelial cells similarly to gliadin: it induced epithelial cell layer permeability, tight junctional protein occludin and ZO-1 distortion and actin reorganization. In high-performance liquid chromatography and mass spectroscopy (HPLC-MS), germinating barley enzymes provided the most efficient degradation of secalin and gliadin peptides and was thus selected for further *in vitro* analysis. After germinating barley enzyme pretreatment, all toxic reactions induced by secalin were ameliorated. We conclude that germinating enzymes from barley are particularly efficient in the degradation of rye secalin. In future, these enzymes might be utilized as a novel medical treatment for coeliac disease or in food processing in order to develop high-quality coeliac-safe food products.

Keywords: Caco-2, coeliac disease, germinating cereal enzymes, innate immunity, permeability

Introduction

The unique composition of cereal prolamins in wheat, barley and rye renders them resistant to gastrointestinal proteolytic enzymes. This is due mainly to a high content of glutamine and proline residues which leads to incomplete degradation of these proteins during normal human digestion [1–3]. Such partial degradation is thought to be one crucial factor in the activation of the immune response in the small-bowel mucosa and the progression of coeliac disease in genetically susceptible people [2,4]. Although coeliac disease has been classified traditionally as a gluten-induced T cell-mediated autoimmune disorder, during the last few years gluten recognition in the mucosal epithelial layer and the role of innate immunity components such as interleukin (IL)-15 [5–9] have claimed increasing attention in discussion of the mechanisms underlying this disease. In particular, modulation of the epithelial barrier is now thought to be a key element in the increased small-intestinal permeability characteristic for coeliac disease [10–13].

Currently the only treatment for coeliac disease is avoidance of gluten prolamins (gliadin, hordein and secalin). Regardless of the fact that oats avenin belongs to the gluten prolamins, there is a large body of evidence for the safety of oat consumption [14–16]. Wheat gliadin has been proved to induce several toxic effects in coeliac disease [6,17–19]. Although rye and barley have also been found to contain several potentially harmful peptides [20,21], which may activate immune reactions in coeliac disease patients, there are only a few studies addressing this issue [22–27]. In practice, rye and barley are excluded from the gluten-free diet mainly in view of their protein homology to wheat.

Because gluten peptides remain fairly intact during gastrointestinal digestion, enzyme supplements have been

proposed as novel therapeutic agents for coeliac disease [2,28–32]. Such supplementary proteases might ensure the complete breakdown of gluten epitopes in advance of their absorption in the small bowel. We have proposed previously that the whole mixture of enzymes isolated from germinating wheat – selected evolutionarily for total cleavage of storage proteins during the germination of kernels – are highly efficient in degrading and detoxifying gliadin peptides [33]. In addition to wheat, other cereals also contain a full range of different enzymes with the potential to accelerate gluten degradation. Interestingly, the proteolytic enzyme EP-B2, isolated from germinating barley seeds, has proved recently to be a particularly promising therapeutic tool for gluten detoxification [32,34–36].

To confirm the assumption that rye should be excluded from coeliac disease patients' diet, we investigated whether rye secalin activates toxic reactions in the small-bowel mucosal epithelial cells as extensively as wheat gliadin. Further, we tested the efficacy of germinating enzymes from oat, wheat and barley to hydrolyse secalin peptides and studied whether the harmful effects elicited by secalin can be reduced by such enzymatic pretreatment.

Materials and methods

Preparation of gliadin and secalin

Gliadin was extracted from wheat (Raisio Oy, Raisio, Finland) and secalin from rye flour (Myllyn Paras Oy, Hyvinkää, Finland). For cell culture experiments both were digested using pepsin (P-6887; Sigma-Aldrich, Seelze, Germany) and trypsin (T-7418, Sigma-Aldrich), as described previously [37]. To abolish the harmful effects exerted by pepsin and trypsin enzymes, the samples were heat-inactivated at >95°C for 10 min. Bovine serum albumin (BSA, A8806; Sigma-Aldrich) was treated similarly and served as a negative control for peptic–tryptic (PT) treatment.

Germination of cereals and comparison of their cleaving capacities

Oat, wheat and barley seeds were germinated in a pilot malting apparatus (Joe White Malting System, Melbourne, Australia) until the most efficient activation of germinating enzymes was achieved at day 8 (data not shown). Germinated grains were isolated and homogenized as described previously [33]. To compare the prolamin degrading capacities of germinating oat, wheat and barley enzymes, crude gliadin and secalin (1 mg/ml) were incubated for 24 h at 37°C in 50 mM Na-acetate buffer, pH 4·0 containing different concentrations of germinated cereal enzymes (0·1–100 μg/ml). The degradation products were analysed by high-performance liquid chromatography and mass spectroscopy (HPLC-MS) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as described below.

To study toxic *in vitro* reactions in intestinal epithelial cells, secalin (6 mg/ml) was incubated similarly with the most effective germinated cereal enzyme preparation (0·3 mg/ml) found during the study and digested with pepsin and trypsin as above.

HPLC-MS and SDS-PAGE of PT–secalin and enzymatically pretreated PT–secalin

HPLC-MS was carried out as described previously [33] using a linear trap quadrupole (LTQ) ion trap mass spectrometer connected to a Surveyor HPLC-MS system (Finnigan, San Jose, CA, USA). To compare the prolamin-cleaving capacity of different germinated grains, several representative m/z signals of full-length gliadin and secalin were selected and their disappearance followed after incubation with increasing concentrations (0·1–100 μg/ml) of grain enzymes. The sum of the signals selected was plotted against enzyme concentration and the data fitted in a standard sigmoidal dose–response curve. Half-maximal effective concentration (EC_{50}) values were calculated from these curves, which give an approximation of the enzyme concentration needed to reduce the amount of full-length prolamin by 50%.

The degradation of barley enzyme-pretreated PT–secalin was monitored using similar HPLC-MS conditions. The size of the peptides formed was approximated by retention times of α-gliadin peptides 12-mer (QLQPFPQPQLPY; New England Peptide, Fitchburg, MA, USA) and 33-mer (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF; New England Peptide), which have been demonstrated to be highly resistant to human digestive enzymes [2,3,38]. Secalin was incubated with or without germinating oat, wheat or barley as described above, whereafter the reaction was stopped by heating (>95°C, 10 min). Subsequently, 2 μl samples were subjected to standard SDS-PAGE analysis on 12% gel.

Cell cultures

Caco-2 cells (passages 19–70, HTB-37; American Type Culture Collection, Rockville, MD, USA) were grown under standard cell culture conditions, maintained in minimum essential medium (Gibco Invitrogen, Paisley, UK) supplemented with 20% fetal bovine serum (FBS; Gibco Invitrogen), 50 U/ml penicillin–streptomycin (Gibco Invitrogen), 1 mM sodium pyruvate (Sigma-Aldrich), 1·5 g/l sodium bicarbonate (Gibco Invitrogen) and 0·1 mM non-essential amino acids (Gibco Invitrogen). The cells were passaged twice a week upon reaching 80% confluence. Before experiments, the culture medium was replaced with starvation media containing 1% fetal bovine serum (FBS), penicillin–streptomycin, sodium pyruvate and non-essential amino acids.

Epithelial cell layer permeability

Caco-2 cells were plated on Millicell Culture (Millipore Corporate, Billerica, MA, USA) and cultured until reaching confluency, measured by Millicell-ERS volt-ohm meter (Millipore Corporate). Once transepithelial resistance (TER) reached 1000 ohms × cm^2, cells were starved overnight and challenged with PT–SA, PT–gliadin, PT–secalin or enzymatically pretreated PT–secalin in a concentration of 1 mg/ml. Thereafter, the recovery of TER was measured once an hour until reaching baseline level within 6 h. The experiments were performed independently in duplicate at least six times.

Immunofluorescence stainings

Expression of the tight junction-associated proteins occludin and ZO-1 was investigated in confluent Caco-2 cell layers, cultured on Transwell polyester membrane inserts (Millipore Corporate) for 5–7 days. The cells were then starved overnight and supplemented with PT-digested BSA, gliadin, secalin or enzymatically pretreated secalin and challenged for 24 h. For stainings, samples were fixed with 4% paraformaldehyde, permeabilized and incubated with mouse monoclonal anti-occludin antibody (1:100; Zymed, San Francisco, CA, USA) or mouse anti-ZO-1 antibody (1:100; Zymed) for 60 min. AlexaFluor 568 goat anti-mouse immunoglobulin (Ig)G (1:1000; Invitrogen, Carlsbad, CA, USA) was used as secondary antibody for 30 min. Stainings were visualized with an Olympus BX60 microscope (Olympus, Hamburg, Germany).

To study actin reorganization, Caco-2 cells were plated onto eight-chamber glass slides (BD Biosciences, Erembodegem, Belgium) and cultured as described previously [33,37]. The cells were then starved and incubated in the presence of study compounds as above. Intracellular F-actin was stained with phalloidin–fluorescein isothiocyanate (1:300; Sigma-Aldrich). The extent of actin cytoskeleton rearrangement was quantified by measuring the cellular edge covered by membrane ruffles as a percentage of the total length of at least 60 different cell clusters in three independent experiments. Calculations were made with analySIS software (Olympus Soft Imaging System GmbH, Munster, Germany).

Statistical analysis

Results are given as means and standard error of means. The two-tailed Mann–Whitney *U*-test was used to assess the statistical significance of differences. *P*-values lower than 0·05 were considered significant.

Results

Comparison of enzymatic degradation of gliadin and secalin

The cleaving capacities of germinating oat, wheat and barley enzymes were compared by incubating gliadin and secalin with several concentrations of enzyme preparations. When calculating the enzyme amounts needed to reduce the level of full-length prolamin by 50% (Table 1), it appeared that the barley enzymes degrade both gliadin and secalin more efficiently than oat and wheat enzymes, even though the differences in EC_{50} values between the enzyme preparations are small. This is evidenced by Fig. 1, showing the peptide profiles of gliadin (Fig. 1a) and secalin (Fig. 1b) after incubation with germinating grain enzymes. Finally, the SDS-PAGE gel of secalin pretreated with oat, wheat or barley enzymes illustrates clearly the superiority of barley enzymes in secalin degradation (Fig. 1c). Of note, heat-inactivated barley enzymes were not able to cleave secalin (Fig. 1c).

Table 1. Efficiency of gliadin and secalin degradation by germinated oat, wheat and barley enzyme preparations.

Enzyme preparation	EC_{50} (μg/ml)	
	Gliadin	Secalin
Oat	4·3 (3·1–5·8)	1·3 (1·2–1·4)
Wheat	2·6 (2·0–3·4)	1·0 (0·9–1·1)
Barley	2·3 (1·6–3·2)	0·7 (0·3–1·5)

Half-maximal effective concentration (EC_{50}) value (μg/ml) represents the mean of enzyme preparation concentrations, needed to reduce the amount of full-length prolamin (1 mg/ml) by 50%. The data are derived from three independent experiments; 95% confidence limits are included in parenthesis.

Because germinating barley enzymes proved most efficacious in degrading prolamins, this enzyme mixture was selected for further experiments. Sequential digestion of PT–secalin with germinating barley enzymes was studied by HPLC-MS analysis (Fig. 1d). Pretreatment of PT–secalin with barley enzymes (red line) resulted in much more efficient secalin degradation than PT–digestion alone (black line). After enzymatic pretreatment, most of the long peptides eluting after 15 min had disappeared, leaving only cleavage products of the pepsin, trypsin and barley enzyme preparation (blue line). Examination of the remaining peptide products by comparison to the standard toxic α-gliadin peptides 12-mer (p57–68) and 33-mer (p56–88) eluted at 16·5 and 23·3 min, showed that germinating barley enzymes efficiently hydrolysed coeliac toxic peptides, as only scant short fragments were left (Fig. 1d).

In vitro toxic reactions in intestinal epithelial cells

Culture media exchange caused an immediate drop of TER in all cell cultures, as described elsewhere [33,39]. However, TER began to recover and reached baseline level in control cultures after 6 h (Fig. 2). In contrast, TER in cultures challenged with PT–secalin failed to recover to the baseline levels similarly to those treated with the positive control, PT–gliadin (Fig. 2). When PT–secalin was pretreated with germinating barley enzymes, the TER recovery paralleled that of negative control cultures supplemented with PT–BSA.

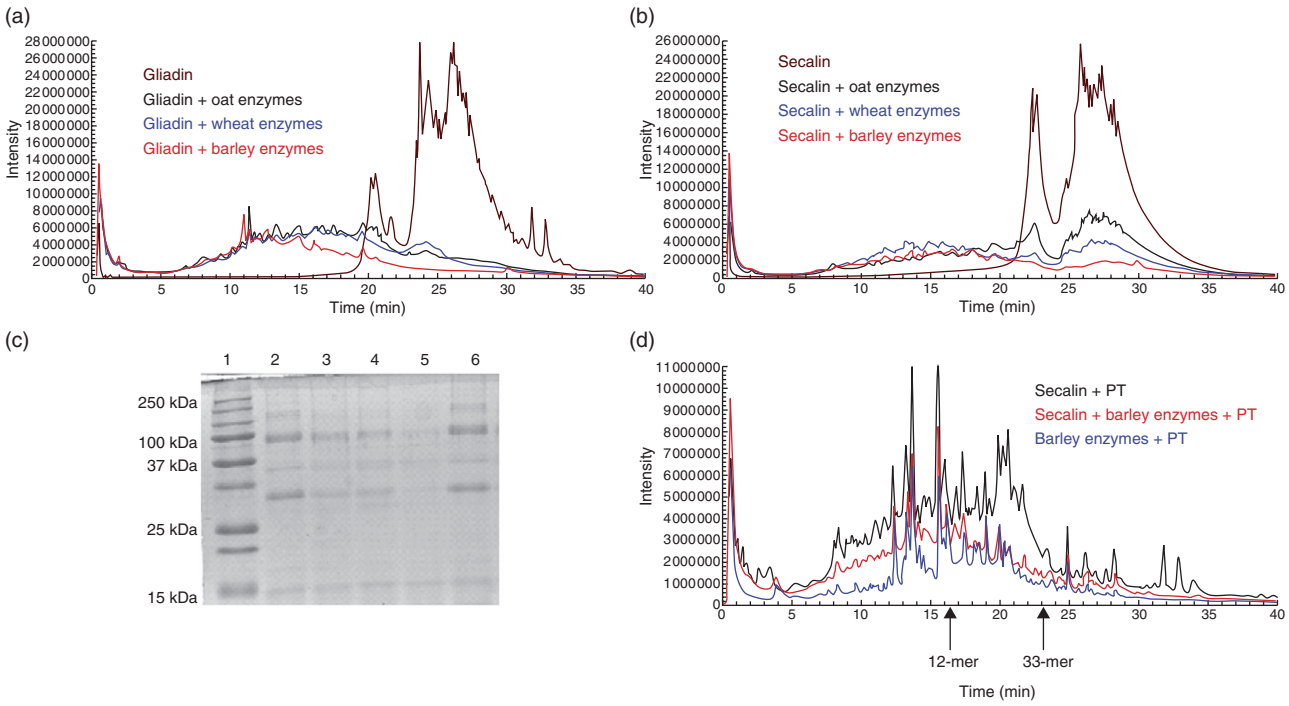

Fig. 1. Enzymatic degradation of gliadin and secalin analysed by high-performance liquid chromatography and mass spectroscopy (HPLC-MS) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). HPLC-MS elution profiles of gliadin (a) and secalin (b) (both 6 mg/ml) incubated with 0·3 mg/ml of oat, wheat and barley enzyme preparations. Full range m/z = 480–2000. (c) SDS-PAGE analysis of secalin (1 mg/ml) degraded by germinated grain preparations (1 µg/ml). Lane 1 = size standard, lane 2 = secalin, lane 3 = secalin + germinating oat enzymes, lane 4 = secalin + germinating wheat enzymes, lane 5 = secalin + germinating barley enzymes, lane 6 = secalin + heat inactivated germinating barley enzymes. (d) HPLC-MS elution profiles of pepsin–trypsin (PT)-digested secalin (black line), PT–secalin pretreated with germinating barley enzymes (red line) and germinating barley enzymes + PT (blue line). Full range m/z = 480–2000. Retention times of the standard peptides 12-mer (16·5 min) and 33-mer (23·3 min) are shown by arrows.

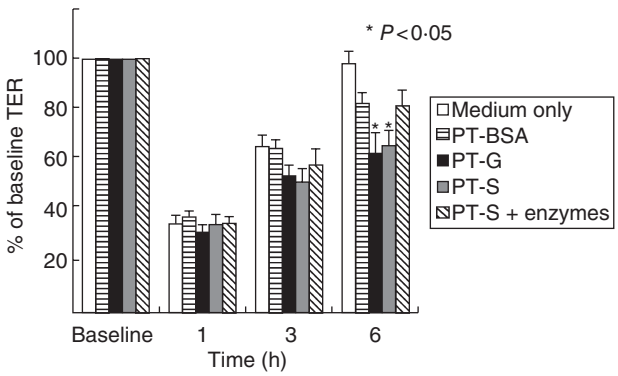

Fig. 2. Transepithelial resistance (TER) in Caco-2 cells cultured with medium only or challenged with pepsin–trypsin-digested bovine serum albumin (PT–BSA), pepsin–trypsin-digested gliadin (PT–G), pepsin–trypsin-digested secalin (PT–S) or germinating barley enzyme-pretreated PT–S (PT–S + enzymes) at different time-points. Data are given as mean percentages of baseline TER values ± standard error of the mean derived from six independent experiments performed in duplicate. Statistical analyses by two-tailed Mann–Whitney *U*-test, where $P < 0{\cdot}05$ (*) was considered statistically significant.

This altered barrier function induced by PT–secalin was accompanied by modulation of distinct tight junctional protein expression. In cultures supplemented with either PT–secalin or PT–gliadin the normal curly appearance of occludin and its associated protein ZO-1 expression was altered and the tight junctions were straightened (Fig. 3a and b). In addition, PT–secalin or PT–gliadin-treated cultures showed marked disruption of the occludin network (Fig. 3a). The pretreatment of PT–secalin with germinating barley enzymes was able to circumvent these harmful effects (Fig. 3a and b).

In another *in vitro* model for gliadin toxicity, addition of PT–secalin induced as extensive membrane ruffles at the edge of the Caco-2 cell layer as PT–gliadin treatment (Fig. 4a and b). This toxic effect of PT–secalin was, however, abolished when PT–secalin was pretreated with germinating barley enzymes. These enzymes themselves had no effects on TER, tight junctional protein expression or actin cytoskeleton rearrangement (data not shown).

Discussion

In this study we have demonstrated that PT–secalin exerts toxic effects similar to those of PT–gliadin in all intestinal

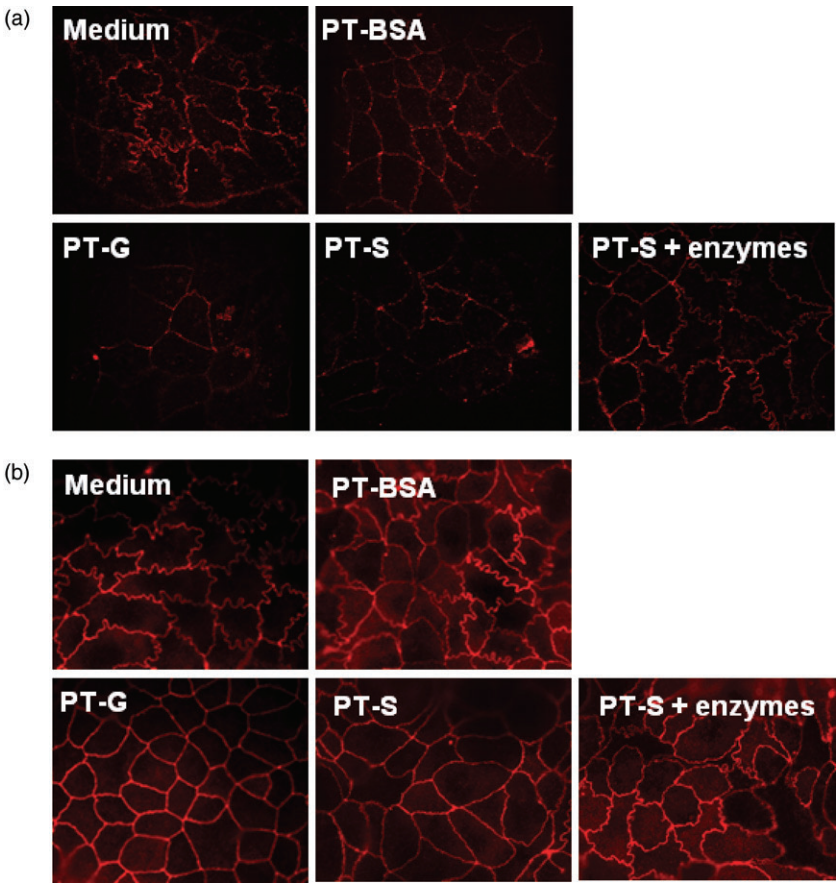

Fig. 3. The appearance of tight junctions in Caco-2 cells cultured with medium only, pepsin–trypsin-digested bovine serum albumin (PT–BSA), pepsin–trypsin-digested gliadin (PT–G), pepsin–trypsin-digested secalin (PT–S) or enzymatically pretreated pepsin–trypsin-digested secalin (PT–S + enzymes). Immunofluorescent staining of occludin (a) and ZO-1 (b). Magnification 100×. Pictures are representative images from experiments performed in duplicate three independent times.

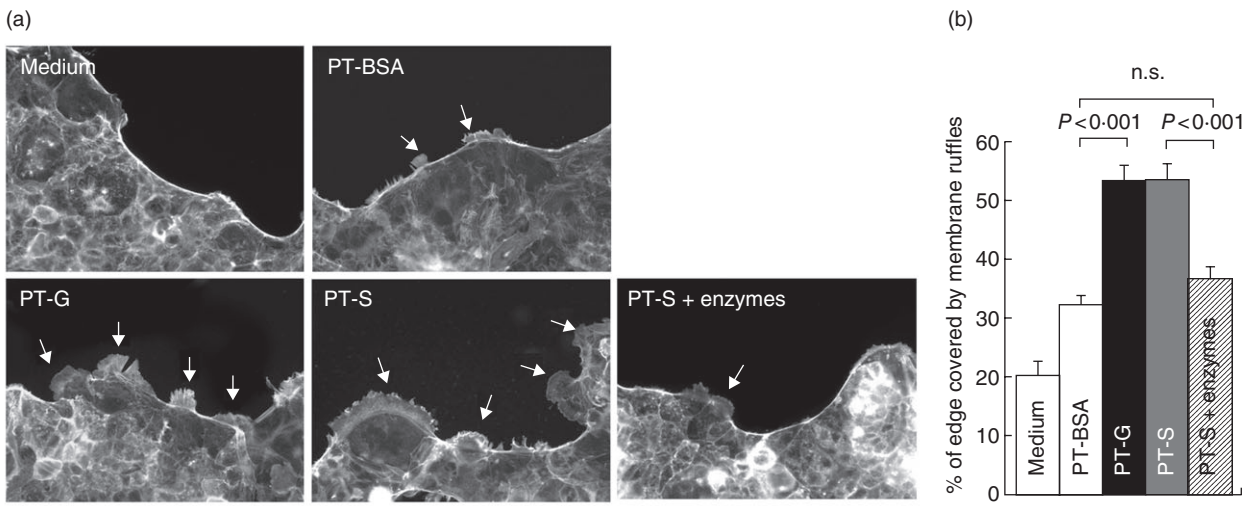

Fig. 4. Actin cytoskeleton rearrangement as membrane ruffling in Caco-2 cells. (a) Representative images of actin membrane ruffling (arrows) in Caco-2 cells cultured with medium only, pepsin–trypsin-digested bovine serum albumin (PT–BSA), pepsin–trypsin-digested gliadin (PT–G), pepsin–trypsin-digested secalin (PT–S) or germinating barley enzyme-pretreated PT–S (PT–S + enzymes). Magnification 20×. (b) Quantitation of ruffle formation was measured as percentage of total cell layer edge length. Data are given as mean values ± standard error of the mean derived from three independent experiments performed in duplicate. Statistical analyses by two-tailed Mann–Whitney *U*-test, where $P < 0{\cdot}05$ was considered statistically significant; n.s.: not significant.

epithelial cell culture experiments *in vitro*: it affected epithelial barrier function by increasing cell monolayer permeability, altered tight junctional protein occludin and ZO-1 expressions and induced extensive actin reorganization at the edges of intestinal epithelial cell clusters. These observations would indicate that secalin contains epitopes which are also able to activate innate immunity pathways in addition to stimulating the T cell-mediated adaptive immunity reactions shown in previous studies [23,27]. These results support the earlier case reports in which it was observed that coeliac disease patients developed symptoms and small-bowel mucosal histological alterations after ingestion of rye [24–26]. Similarly, Kieffer and coworkers [22] demonstrated that coeliac patient serum samples contained antibodies which recognize rye and barley in addition to wheat, suggesting that the immunological response of coeliac disease patients can be activated by all these cereals, including rye.

During recent years, discussion has arisen of alternative therapeutic strategies for coeliac disease. Several external enzymes have been proposed to detoxify gluten epitopes [2,31,34,40]. However, the complexity of the wheat gliadin molecule has made the procedure a very challenging task [28,41]. For the full cleavage of gluten peptides a combination of different enzymes, rather than one specific protease, is probably needed [32,35,42]. To this end, we have introduced previously a natural means of gluten detoxification using a whole mixture of wheat own enzymes, intended evolutionarily for the total digestion of wheat storage proteins during the germination of wheat kernels [33]. In the study in question, germinating wheat proteases cleaved gliadin peptides efficiently into short fragments, whereafter the toxicity of gliadin was diminished markedly in different *in vitro* models. Similarly, in the current study, germinating cereal enzymes proved particularly powerful in hydrolysing secalin. However, instead of using germinating wheat enzymes, we now compared the efficacy of three different cereal (oat, wheat and barley) enzymes to achieve the maximum cleavage of secalin peptides. Of these, barley enzymes were superior in cleaving gliadin and secalin (Table 1, Fig. 1). Furthermore, when testing the toxic reactions of PT–secalin pretreated with germinating barley enzymes we observed diminished toxicity in all Caco-2 cell culture experiments compared to unprocessed PT–secalin.

In the current study we used the Caco-2 cell line as a model to investigate the toxicity of gluten-containing cereals. As Caco-2 cells originate from the colon, they have some limitations when studying coeliac disease. For example, it has been published that Caco-2 cells do not express a wide variety of different proinflammatory cytokines [43]. IL-15 is a central cytokine in coeliac disease-related innate immune reactions [5,7–9]; however, it is secreted poorly in the cultured intestinal cells as well as organ culture biopsy samples of coeliac disease patients, as shown in previous studies [5,9]. Similarly, we were unable to measure the amount of IL-15 in the Caco-2 culture medium even in the presence of gliadin (data not shown).

Of note, it was observed that PT–BSA had minor toxic effects in all cell culture experiments when compared to cells cultured without any supplementation, as also seen in earlier studies [33,37]. This is due probably to remaining amounts of pepsin and trypsin in the samples in spite of heat inactivation. In earlier studies it has been demonstrated that contaminating trypsin might alter gliadin peptide-binding characteristics by direct binding to intestinal epithelial cell brush border membranes [44]. Therefore, the inclusion of PT-treated control in the experiments is important to reveal the true effect exerted by toxic compounds. In spite of these limitations, the Caco-2 cell line has been used widely in coeliac disease research, especially when studying the toxicity of gliadin [11,12,37,45,46].

We conclude that rye secalin activates toxic reactions *in vitro* similarly to wheat gliadin and should thus be excluded from the diet of coeliac disease patients, as suggested previously [23,25–27]. However, the taste and nutritional value of rye is highly appreciated in some countries and therefore coeliac-safe processed rye-based products are called for. Our present results indicate that the whole mixture of germinating barley enzymes is highly efficient in the elimination of residual toxic peptides of rye secalin and could therefore be of value in the medical treatment of coeliac disease or in the food industry in developing novel coeliac-safe food products in the future.

Acknowledgements

The authors thank Mrs Jaana Leskinen and Mr Jorma Kulmala for technical assistance. The Celiac Disease Study Group and the current work have been financially supported by the Tampere Graduate School in Biomedicine and Biotechnology, the Academy of Finland, the Pediatric Research Foundation, the Competitive Research Funding of the Tampere Hospital District, the Finnish Celiac Society, the Finnish Cultural Foundation, the Sigrid Juselius Foundation and the European Comission (contract number MRTN-CT-2006-036032).

Disclosure

None.

References

1 Bruce G, Woodley JF, Swan CH. Breakdown of gliadin peptides by intestinal brush borders from coeliac patients. Gut 1984; **25**:919–24.

2 Shan L, Molberg O, Parrot I *et al.* Structural basis for gluten intolerance in celiac sprue. Science 2002; **297**:2275–9.

3 Hausch F, Shan L, Santiago NA, Gray GM, Khosla C. Intestinal

digestive resistance of immunodominant gliadin peptides. Am J Physiol Gastrointest Liver Physiol 2002; **283**:G996–G1003.
4 Sollid LM. Coeliac disease: dissecting a complex inflammatory disorder. Nat Rev Immunol 2002; **2**:647–55.
5 Maiuri L, Ciacci C, Auricchio S, Brown V, Quaratino S, Londei M. Interleukin 15 mediates epithelial changes in celiac disease. Gastroenterology 2000; **119**:996–1006.
6 Maiuri L, Ciacci C, Ricciardelli I *et al.* Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. Lancet 2003; **362**:30–7.
7 Di Sabatino A, Ciccocioppo R, Cupelli F *et al.* Epithelium derived interleukin 15 regulates intraepithelial lymphocyte Th1 cytokine production, cytotoxicity, and survival in coeliac disease. Gut 2006; **55**:469–77.
8 Yokoyama S, Watanabe N, Sato N *et al.* Antibody-mediated blockade of IL-15 reverses the autoimmune intestinal damage in transgenic mice that overexpress IL-15 in enterocytes. Proc Natl Acad Sci USA 2009; **106**:15849–54.
9 Mention JJ, Ben Ahmed M, Begue B *et al.* Interleukin 15: a key to disrupted intraepithelial lymphocyte homeostasis and lymphomagenesis in celiac disease. Gastroenterology 2003; **125**:730–45.
10 Schulzke JD, Schulzke I, Fromm M, Riecken EO. Epithelial barrier and ion transport in coeliac sprue: electrical measurements on intestinal aspiration biopsy specimens. Gut 1995; **37**:777–82.
11 Sander GR, Cummins AG, Henshall T, Powell BC. Rapid disruption of intestinal barrier function by gliadin involves altered expression of apical junctional proteins. FEBS Lett 2005; **579**:4851–5.
12 Lammers KM, Lu R, Brownley J *et al.* Gliadin induces an increase in intestinal permeability and zonulin release by binding to the chemokine receptor CXCR3. Gastroenterology 2008; **135**:194–204 e3.
13 Tripathi A, Lammers KM, Goldblum S *et al.* Identification of human zonulin, a physiological modulator of tight junctions, as prehaptoglobin-2. Proc Natl Acad Sci USA 2009; **106**:16799–804.
14 Janatuinen EK, Pikkarainen PH, Kemppainen TA *et al.* A comparison of diets with and without oats in adults with celiac disease. N Engl J Med 1995; **333**:1033–7.
15 Hogberg L, Laurin P, Falth-Magnusson K *et al.* Oats to children with newly diagnosed coeliac disease: a randomised double blind study. Gut 2004; **53**:649–54.
16 Koskinen O, Villanen M, Korponay-Szabo I, Lindfors K, Maki M, Kaukinen K. Oats do not induce systemic or mucosal autoantibody response in children with coeliac disease. J Pediatr Gastroenterol Nutr 2009; **48**:559–65.
17 Sturgess R, Day P, Ellis HJ *et al.* Wheat peptide challenge in coeliac disease. Lancet 1994; **343**:758–61.
18 Anderson RP, Degano P, Godkin AJ, Jewell DP, Hill AV. *In vivo* antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. Nat Med 2000; **6**:337–42.
19 Stenman SM, Lindfors K, Korponay-Szabo IR *et al.* Secretion of celiac disease autoantibodies after *in vitro* gliadin challenge is dependent on small-bowel mucosal transglutaminase 2-specific IgA deposits. BMC Immunol 2008; **9**:6.
20 Wieser H. The precipitating factor in coeliac disease. Baillières Clin Gastroenterol 1995; **9**:191–207.
21 Vader LW, Stepniak DT, Bunnik EM *et al.* Characterization of cereal toxicity for celiac disease patients based on protein homology in grains. Gastroenterology 2003; **125**:1105–13.
22 Kieffer M, Frazier PJ, Daniels NW, Coombs RR. Wheat gliadin fractions and other cereal antigens reactive with antibodies in the sera of coeliac patients. Clin Exp Immunol 1982; **50**:651–60.
23 Kilmartin C, Wieser H, Abuzakouk M, Kelly J, Jackson J, Feighery C. Intestinal T cell responses to cereal proteins in celiac disease. Dig Dis Sci 2006; **51**:202–9.
24 Baker PG, Read AE. Oats and barley toxicity in coeliac patients. Postgrad Med J 1976; **52**:264–8.
25 Anand BS, Piris J, Truelove SC. The role of various cereals in coeliac disease. Q J Med 1978; **47**:101–10.
26 Dicke WK, Weijers HA, Van De Kamer JH. Coeliac disease. II. The presence in wheat of a factor having a deleterious effect in cases of coeliac disease. Acta Paediatr 1953; **42**:34–42.
27 Bracken SC, Kilmartin C, Wieser H, Jackson J, Feighery C. Barley and rye prolamins induce an mRNA interferon-gamma response in coeliac mucosa. Aliment Pharmacol Ther 2006; **23**:1307–14.
28 Shan L, Marti T, Sollid LM, Gray GM, Khosla C. Comparative biochemical analysis of three bacterial prolyl endopeptidases: implications for coeliac sprue. Biochem J 2004; **383**:311–18.
29 Gass J, Ehren J, Strohmeier G, Isaacs I, Khosla C. Fermentation, purification, formulation, and pharmacological evaluation of a prolyl endopeptidase from *Myxococcus xanthus*: implications for celiac sprue therapy. Biotechnol Bioeng 2005; **92**:674–84.
30 Sollid LM, Khosla C. Future therapeutic options for celiac disease. Nat Clin Pract Gastroenterol Hepatol 2005; **2**:140–7.
31 Stepniak D, Spaenij-Dekking L, Mitea C *et al.* Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. Am J Physiol Gastrointest Liver Physiol 2006; **291**:G621–9.
32 Siegel M, Bethune MT, Gass J *et al.* Rational design of combination enzyme therapy for celiac sprue. Chem Biol 2006; **13**:649–58.
33 Stenman SM, Venalainen JI, Lindfors K *et al.* Enzymatic detoxification of gluten by germinating wheat proteases: implications for new treatment of celiac disease. Ann Med 2009; **41**:390–400.
34 Gass J, Vora H, Bethune MT, Gray GM, Khosla C. Effect of barley endoprotease EP-B2 on gluten digestion in the intact rat. J Pharmacol Exp Ther 2006; **318**:1178–86.
35 Gass J, Bethune MT, Siegel M, Spencer A, Khosla C. Combination enzyme therapy for gastric digestion of dietary gluten in patients with celiac sprue. Gastroenterology 2007; **133**:472–80.
36 Tye-Din JA, Anderson RP, Ffrench RA *et al.* The effects of ALV003 pre-digestion of gluten on immune response and symptoms in celiac disease *in vivo*. Clin Immunol 2010; **134**:289–95.
37 Lindfors K, Blomqvist T, Juuti-Uusitalo K *et al.* Live probiotic *Bifidobacterium lactis* bacteria inhibit the toxic effects induced by wheat gliadin in epithelial cell culture. Clin Exp Immunol 2008; **152**:552–8.
38 Arentz-Hansen H, Korner R, Molberg O *et al.* The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. J Exp Med 2000; **191**:603–12.
39 Li N, DeMarco VG, West CM, Neu J. Glutamine supports recovery from loss of transepithelial resistance and increase of permeability induced by media change in Caco-2 cells. J Nutr Biochem 2003; **14**:401–8.
40 Mitea C, Havenaar R, Drijfhout JW, Edens L, Dekking L, Koning F. Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: implications for coeliac disease. Gut 2008; **57**:25–32.

41 Matysiak-Budnik T, Candalh C, Cellier C *et al.* Limited efficiency of prolyl-endopeptidase in the detoxification of gliadin peptides in celiac disease. Gastroenterology 2005; **129**:786–96.
42 Ehren J, Moron B, Martin MT, Bethune E, Gray GM, Khosla C. A food-grade enzyme preparation with modest gluten detoxification properties. PLoS ONE 2009; **4**:e6313.
43 Jung HC, Eckmann L, Yang SK *et al.* A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J Clin Invest 1995; **95**:55–65.
44 Bolte G, Osman A, Mothes T, Stern M. Peptic–tryptic digests of gliadin: contaminating trypsin but not pepsin interferes with gastrointestinal protein binding characteristics. Clin Chim Acta 1996; **247**:59–70.
45 Ciccocioppo R, Finamore A, Ara C, Di Sabatino A, Mengheri E, Corazza GR. Altered expression, localization, and phosphorylation of epithelial junctional proteins in celiac disease. Am J Clin Pathol 2006; **125**:502–11.
46 Barone MV, Gimigliano A, Castoria G *et al.* Growth factor-like activity of gliadin, an alimentary protein: implications for coeliac disease. Gut 2007; **56**:480–8.