Celiac disease : towards new therapeutic modalities

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Chapter 5

Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: implications for celiac disease

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ABSTRACT

Celiac disease is caused by an immune response to gluten. As gluten proteins are proline-rich they are resistant to enzymatic digestion in the gastrointestinal tract, a property that likely contributes to the immunogenic nature of gluten. In this study we have determined the efficiency of gluten degradation by a post- proline cutting enzyme, prolyl endoprotease from *Aspergillus niger* (AN-PEP), in a dynamic system that closely mimics the human gastrointestinal tract (TIM-system). Two experiments were performed. In the first, a slice of bread was processed in the TIM system with and without co-administration of AN-PEP. In the second, a standard fast food menu was used. Samples of the digesting meals were taken from the stomach, duodenum, jejunum and ileum compartments at time zero until four hours after the start of the experiment. In these samples the levels of immunogenic peptides from gliadins and glutenins were assessed by monoclonal antibody based competition assays, Western blot analysis and proliferation T-cell assays. AN-PEP accelerated the degradation of gluten in the stomach compartment to such an extent that hardly any gluten reached the duodenum compartment. AN-PEP is capable of accelerating the degradation of gluten in a gastrointestinal system that closely mimics *in vivo* digestion. This implies that co-administration of AN-PEP with a gluten containing meal might eliminate gluten toxicity, thus offering patients the possibility to (occasionally) abandon their strict gluten-free diet.

INTRODUCTION

Celiac disease (CD) is a small intestinal disorder characterized by an abnormal immune response to gluten proteins. In CD patients ingestion of gluten evokes an immune response in the small intestine that eventually results in T cell infiltration and flattening of the mucosa (1). Patients experience malabsorption, diarrhea and failure to thrive, leading to fatigue, osteoporosis and/or neurological symptoms. Gluten proteins are the storage proteins of wheat and contain high percentages of proline (20%) and glutamine residues (38%). Because of their unusual high content of proline (2) gluten is poorly degraded by enzymes present in the gastrointestinal tract (GI-tract). After ingestion, partially degraded gluten proteins reach the small intestine. Such fragments are good substrates for the enzyme tissue transglutaminase which can convert the amino acid glutamine in gluten into the negatively charged glutamic acid. These modified gluten fragments can bind with high affinity to the disease-associated HLA-DQ2 or HLA-DQ8 molecules and induce inflammatory T cell responses (3-6).

Gluten is composed of two different protein families, the gliadins and glutenins. The gliadins can be further subdivided in α-, β-, γ-, and ω-gliadins and the glutenins into low molecular weight (LMW-) and high molecular weight (HMW-) glutenins. T cell stimulatory epitopes have been identified in all these proteins (7-13),(8;14;15).

For patients with CD the only cure is a lifelong strict gluten-free diet (GFD), which in practice excludes all foods that contain wheat, barley, rye and oats. Because of the widespread use of gluten and gluten-like proteins in food products, a GFD has a great impact on the lifestyle of CD patients. For this reason the search for new treatments, which are compatible with a normal social lifestyle, is of great importance. In this respect several lines of research have been proposed. Studies are performed in which wheat varieties are screened for the level of T cell stimulatory epitopes. Wheat varieties with a low toxicity might form the basis for future breeding programs to generate wheat varieties suitable for generation of food products that can be consumed by CD patients (16-18). Another option is the use of enzymes that degrade the proline-rich gluten molecules before they reach the small intestine. In this context prolyl oligopeptidases were investigated. Such enzymes are not only effective in degrading gluten, the generation of smaller gluten fragments also improves the digestibility of gluten proteins by rendering them more accessible to brush border enzymes (19-25). However, to avoid T cell recognition gluten must be degraded before it reaches the small intestine and the prolyl oligopeptidases investigated are not active under the conditions found in the stomach. These enzymes are thus not suitable for oral supplementation as an alternative treatment for CD.

Recently, we described a prolyl endoprotease from *Aspergillus niger* (AN-PEP) (39). This enzyme was found to efficiently degrade gluten peptides and intact gluten proteins. Moreover, the pH optimum of the enzyme is compatible with that found in the stomach and the enzyme is resistant to degradation by pepsin. These results indicate that this enzyme might be suitable for oral supplementation to degrade gluten proteins in food before they reach the small intestine. To test this we determined the efficiency of gluten degradation under near *in vivo* conditions. To predict the efficacy of enzymes and drugs for therapeutic use in the GI-tract of humans, a dynamic, multi-compartimental *in vitro* system was developed (26). Validation studies demonstrated that this system, called TIM (TNO gastro-Intestinal Model), allows a close simulation of *in vivo* dynamic physiological processes that occur within the lumen of the stomach and small intestine of humans and reliably predicts *in vivo* data (27-29). The system is fully computerized and based on parameters obtained from data of healthy volunteers. The main parameters of digestion, such as pH, body temperature, peristaltic mixing and transit, salivary, gastric, biliary, and pancreatic secretions, as well as absorption of small molecules (e.g. nutrients and drugs) and water are simulated. GI passage and successive conditions can be adjusted in order to mimic parameters in humans at different stages (infant, adult, and elderly), different food intakes and physiological or pathological conditions (such as gastric hyperacidity or pancreatic failure) (29;30).

The result of the present study demonstrates that gluten degradation was strongly accelerated by the presence of AN-PEP in the stomach compartment. AN-PEP was capable of degrading all T cell stimulatory epitopes of gluten tested for to levels below the detection limit of the methods used. Co-administration of AN-PEP with a gluten containing meal may thus be a feasible approach to detoxify gluten before it can do harm in the small intestine of CD patients. This may offer patients an alternative to the strict GFD and thereby improve their quality of life.

MATERIALS AND METHODS

Enzyme prolyl endoprotease from *Aspergillus niger* (AN-PEP)

Prolyl endoprotease from *Aspergillus niger* (AN-PEP) was produced and purified by DSM Food Specialties (Delft, The Netherlands) according to established procedures (31). Activity of the protein is expressed in Proline Protease Units (PPU). A PPU is defined as the quantity of enzyme that releases 1 µmol of p-nitroanilide per minute at 37^0 C in a citrate/disodium phosphate buffer pH 4.6 and at a substrate concentration of 0.37 mM Z-Gly-Pro-pNA (Bachem, Bubendorf, Switzerland) and represents 10 mg of pure protein. The reaction products were monitored spectrophotometrically at 405 nM.

TIM experiments

The TIM has been described in detail previously (26-30). This model has compartments for the stomach, duodenum, jejunum and ileum (Figure 1). Each compartment has a flexible inner wall surrounded by water at 37º C. Changing water pressure squeezes the walls to simulate peristaltic mixing of the food with the ‘secreted’ electrolytes and enzymes. The transport of the chyme is regulated by the peristaltic valves that connect the successive compartments. Using various sensors in the compartments, the pH values, temperature, volumes, and pressure, as well as the gastric emptying and small intestinal passage of the food are computer-controlled according to pre-set curves. For the present experiments all parameters in TIM were adjusted to simulate the average physiological conditions in the GI tract of young healthy adults after the intake of the type of meal as described below. During 2.5 h the gastric content was gradually delivered into the small intestine via the ‘pyloric valve’. After 5 h approximately 80% of the small-intestinal content was gradually delivered into the 'large intestine' (sampling bottle) via the ‘ileo-caecal valve’.

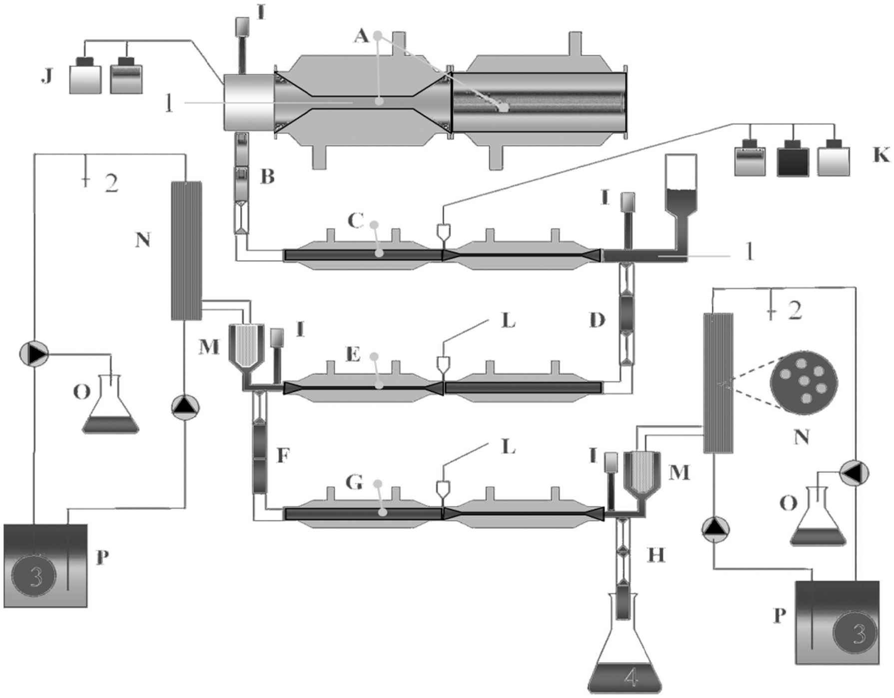

Figure 1. Schematic diagram of the dynamic, multi-compartmental model of the stomach and small intestine (TIM system).

A. stomach compartment; B. pyloric sphincter; C. duodenum compartment; D. peristaltic valve; E. jejunum compartment; F. peristaltic valve; G. ileum compartment; H. ileo-caecal sphincter; I. pH electrodes; J. pancreatin, bicarbonate; L. secretion of bicarbonate to control the intestinal pH; M. pre-filter system; N. hollow fibre semi-permeable membrane system; O. water absorption system; P. closed dialysing system.

For 2.5 hours the gastric content was mixed by peristaltic movements with added saliva and gastric juice (NaCl (4.8 g/L), KCl (2.2 g/L), CaCl2 (0.22 g/L) and NaHCO3 (1.5 g/L) with pepsin 500 KU/L (Sigma, P-7012) and gradually delivered into the small intestine via the ‘pyloric valve'. Throughout the transit in the duodenum compartment the content was mixed by peristaltic movements with bicarbonate up to pH 6.4 ± 0.2 and with ‘secreted’ bile and pancreatic juice. Also in the jejunum and ileum compartments the content was mixed with bicarbonate up to pH 6.8 ± 0.2 and pH 7.1 ± 0.2, respectively. The digested compounds were dialyzed continuously from the jejunum and ileum compartments via semi-permeable hollow fiber membrane systems.

Two experiments were performed. In the first experiment 70 g of white bread (containing 5 g of gluten) and 110 ml drinking water was homogenized together with 110 ml of artificial saliva in the absence or presence of AN-PEP (200 mg pure enzyme/g protein). After 40 seconds of homogenization the mixture was added to the stomach compartment of the TIM-system containing 10 ml of simulated gastric juice and the experiment was started. In the second experiment a quarter of a commercial fast food menu consisting of a bread bun, a hamburger, ketchup, French fries, and supplemented with additional bread (50 g in total), was homogenized with 110 ml soda and 110 ml of artificial saliva in the presence or absence of AN-PEP (200 mg pure enzyme/g protein) and introduced in the TIM-system.

Sampling and analysis

During the transit of the homogenized food products through the compartments of the TIM-system, samples of 2 ml were taken at time points: 0, 15, 30, 45, 60, 90, 120, 150, 180 and 240 minutes from the stomach, duodenum, jejunum and ileum compartments. The samples were snap frozen in dry ice to stop enzymatic activity.

Before analysis, the samples were thawed and AN-PEP activity was stopped by increasing the pH to 11-12 using 1 M NaOH, followed by neutralization with 1M HCl. Hereafter the samples were kept at 85° C for 10 minutes to inactivate any residual enzymatic activity. Identical volumes from each sample were centrifuged for 10 minutes at 14.000 rpm to separate the water-soluble and water-insoluble components. The water-insoluble fractions were solubelized in the same volume of 6x protein sample buffer (60% glycerol, 300 mM Tris (pH 6.8), 12 mM EDTA pH 8.0, 12 % SDS, 864 mM 2-mercaptoethanol, 0.05% bromophenol blue). From these solutions 2 µl from each sample was used for the protein and Western blot analysis.

Synthetic peptides

Peptides were synthesized as described previously (32).

MAb against the Glia-α20, Glia-α9, Glia-γ1, LMW and HMW glutenin T cell stimulatory epitopes

The specificity and the IgG subclass of the antibodies used in this study are presented in Table 1.

For the generation of a mAb specific for the α-gliadin derived T cell stimulatory epitope Glia-α20, mice were immunized with peptides chemically cross-linked to tetanus toxoid (TTd-DDDXPFRPQQPYPQP-amide). Fusion and screening of the hybridomas was performed as described (32). The minimal epitope of the anti-Glia-α20 mAb (FRPQQPYP) was determined using a set of partially overlapping 17-mer synthetic peptides.

Table 1. Overview of mAbs specific for T cell stimulatory epitopes involved in celiac disease.

Specificity	**T- cell epitope**	**mAb specificity**	**mAb subclass**
α-Gliadin	Glia -a9	Q**LQPFPQPQ**LPY	IgG1 (k light chain)
	Glia-a20	QPQP**FRPQQPYPQ**PQP	IgG1 (k light chain)
γ-Gliadin	Glia-γ1	QPQQPQQSFPQ**QQRPFI**	IgG1 (k light chain)
LMW- glutenin	Glt-156	Q**PPFSQQ**QQSPFSQ* QPPFSQQQ**QSPFS**Q**	IgG3 (k light chain) IgG1 (k light chain)
HMW-glutenin		QQ**GYYPTSP**QQSG	IgG1 (k light chain)

* antibody used for staining western blots

** antibody used in competition assays experiments

Competition assays for the quantitative detection of T cell stimulatory epitopes

Competition assays were performed as described earlier (16;32). Microtitre plates (Nunc Maxisorb, Immunoplate; Nunc, Copenhagen, Denmark) were incubated overnight with 2–5 µg/ml mAb in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.2, at room temperature. Plates were washed in PBS/0.02% Tween-20 and residual binding sites were blocked with PBS/ 1% skim milk powder (Fluka, Zwijndrecht, the Netherlands). Of the gluten containing samples, different dilutions were made in a buffer containing 50 mM Na_2HPO_4/NaH_2PO_4 pH 7.0, 150 mM NaCl, 0.1% Tween-20/ 0.1% skim milk and a protease inhibitor cocktail (Complete, Roche Diagnostics GmbH, Penzberg, Germany). For the detection of gliadins these were mixed with either a biotinylated Glia-α9 or Glia-γ1 gliadin T cell epitope encoding peptides (32). For the detection of the Glia-α20 T cell stimulatory epitope a biotinylated peptide encoding the Glia-α20 epitope was used as the indicator peptide (Bio-XKAKAKAKAXPFRPQQPYPQP-amide). For quantification, a standard curve was made using the Prolamine working group (PWG) gliadin reference material (33) in the concentration range 10 µg/ml-10 ng/ml mixed with biotinylated indicator peptides. The assays, specific for the detection of T cell stimulatory epitopes of LMW glutenin, were calibrated using a 25-mer synthetic peptide as standard that contains the Glt-156 epitope (16). The HMW-glutenin specific assay was calibrated using a chymotrypsin digest of six purified HMW-glutenin proteins (kindly provided by P. Shewry, Rothamsted Research, Hampenden, United Kingdom) Both standards were used in a concentration range from 1 µg/ml-2 ng/ml.

The mixtures were incubated on plates for 1.5 hours at room temperature. Next, plates were washed and incubated for 30 minutes with streptavidin conjugated horseradish peroxidase in PBS/ 0.1% skim milk.

Thereafter, bound peroxidase was visualised by incubation with a solution of 3’, 3’, 5, 5’-tetramethylbenzidine (Sigma-Aldrich Zwijndrecht, the Netherlands). Finally, absorbance at 450 nm was read on a Multiscan plate reader (Wallac, Turku, Finland).

Protein analysis by 1D SDS-PAGE and Western blotting

To determine the level of T cell stimulatory epitopes present in the water-insoluble fractions, these were solubilized in 6x protein sample buffer (60% glycerol, 300 mM Tris (pH 6.8), 12 mM EDTA pH 8.0, 12 % SDS, 864 mM 2-mercaptoethanol, 0.05% bromophenol blue). The samples were separated on a 12.5% SDS-PAGE gels for detection of gliadins and LMW glutenins and on 10% SDS-PAGE gels for detection of HMW glutenins. The proteins were visualized either directly using Imperial Protein Stain (Pierce, Rockford IL, USA), or after transfer to PVDF membranes with the mAbs specific for stimulatory T-cell epitopes from α- and γ-gliadin (32)(and this study) and HMW- and LMW glutenins (16).

T-cell proliferation assay

For the T cell proliferation assays the samples were treated with amylglucosidase and trypsin. Next the samples were incubated O/N at 37°C with guinea pig tissue transglutaminase (200 ug/ml) in the presence of 10 mM CaCl2. Proliferation assays were performed in triplicate in 150 µl Iscove's Dulbecco's medium (BioWhittaker, Vervier Belgium) supplemented with 10% human serum in 96-well flat bottom plates (Costar, Corning Inc., Corning, USA) using 10^4 gluten specific T-cells stimulated with 10^5 irradiated HLA-DQ2-matched allogenic PBMCs (3000 RAD). Of the samples three different amounts of the samples were tested: 0.5 ul, 0.16 ul and 0.05 ul respectively. These amounts were shown to be none toxic for the T-cells. After 48 h incubation at the 37°C, cultures were pulsed with 0.5 µCi of ^{3}H-thymidine, harvested 18h later and the thymidine incorporation was quantified with a liquid scintillation counter (1205 Betaplate Liquid Scintillation Counter; LKB Instruments, Gaithersburg, Maryland, USA).

RESULTS

Digestion of bread and a fast food menu in the TIM-system

To study the effect of AN-PEP on the degradation of gluten proteins in the GI-tract, two experiments were performed. These experiments were designed to represent a small meal (e.g. breakfast) and a more complex meal (e.g. dinner). In the first experiment 70 g of white bread was homogenized together with artificial saliva in the presence or absence of AN-PEP. In the second experiment a quarter of a fast food menu consisting of a sandwich, a hamburger, ketchup, French fries and a glass of soda was homogenized with artificial saliva in the presence or absence of AN-PEP. Since the quantity of bread in this menu was relatively low (16 g), extra bread was added to a total amount of 50 g. After homogenization the mixtures were introduced in the TIM-system, mimicking the human GI-tract (Figure 1). Samples were collected at time points 0, 15, 30, 45, 60, 90, 120, 150, 180 and 240 minutes after the start of the experiment from the stomach, duodenum, jejunum and ileum compartments.

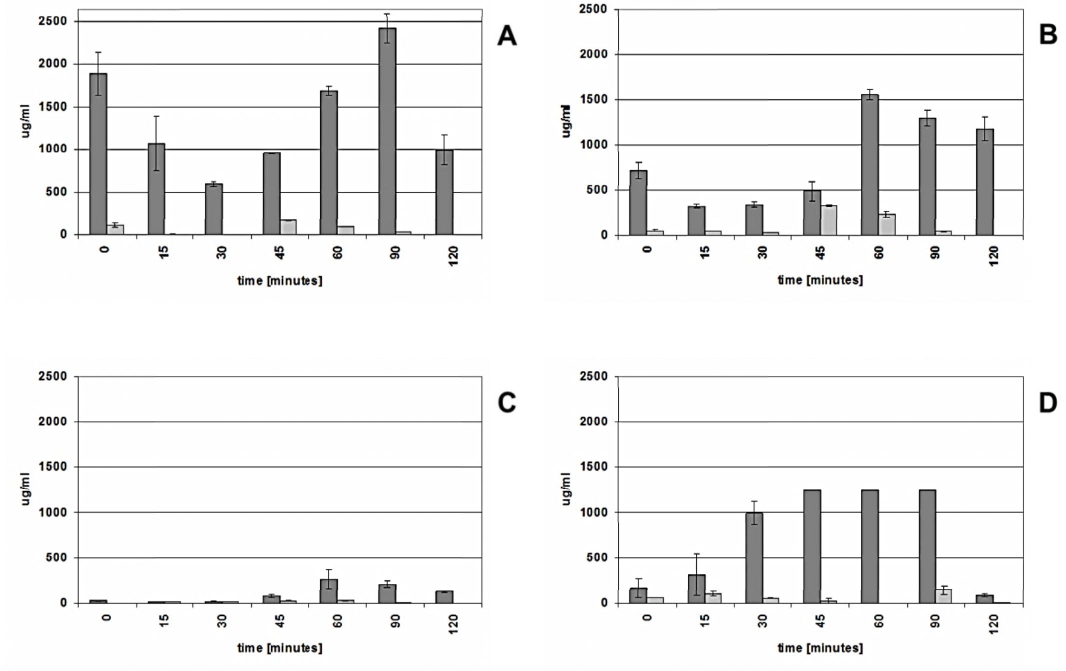

Figure 2. The presence of T cell stimulatory epitopes of α- and γ-gliadin, HMW- and LMW-glutenin in the water-soluble fractions of the white bread digested in the TIM system.

70 g of bread was digested in the TIM system in the presence (gray bars) or absence (black bars) of AN-PEP. At the indicated time points samples were taken from the stomach compartment. The samples were divided in water-soluble and water-insoluble material and the level of T cell stimulatory epitopes in the water-soluble material was determined with mAb based competition assays (16;32)(this study). Levels of the lia-α9 (A), Glia-γ1 (B), HMW-glutenin (C) and LMW-glutenin (D) epitopes in the water-soluble part of stomach fractions.

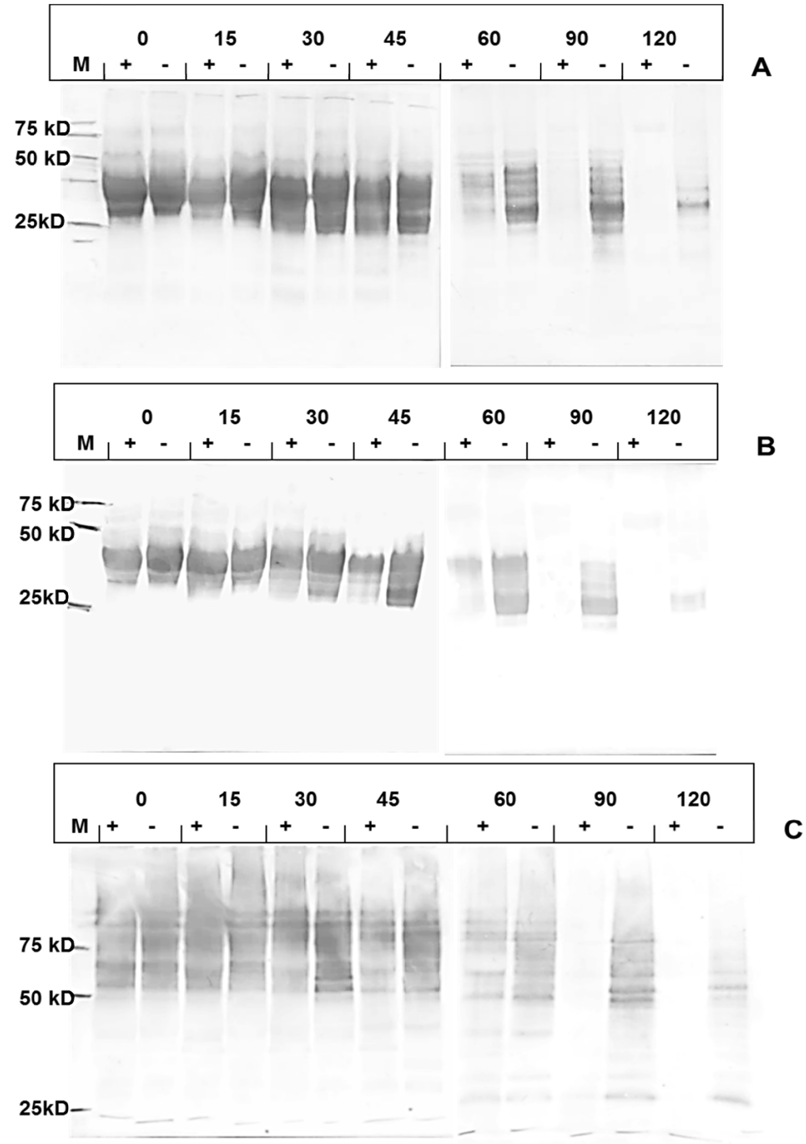

Figure 3. The presence of T cell stimulatory epitopes of α-, γ-gliadin and HMW- glutenin in the water-insoluble fractions of the white bread digested in the TIM system.

70 gram of bread was digested in the TIM system in the presence (+) or absence (-) of AN-PEP. At the indicated time points samples were taken from the stomach compartment and the proteins present in the water-insoluble part of the fraction were separated by 1D SDS-PAGE. After transfer to a PVDF membrane the presence of proteins containing T cell stimulatory epitopes of Glia-α9 (A), Glia-γ1 (B) and HMW-glutenin (C) were visualized using epitope specific mAb (16;32).

In order to study the degradation of gluten in the TIM-experiments, three types of assays were performed. The presence of gluten peptides in the water-soluble fractions was measured by monoclonal antibody (mAb) based competition assays specific for T cell stimulatory peptides present in gliadin (Glia-α9, Glia-α20, Glia-γ1) and LMW- and HMW-glutenin (16;32) (this study). The presence of T cell stimulatory epitopes from gluten in the water-insoluble fractions was determined by Western blot analysis. Moreover samples of the fast food menu taken at 60 minutes after the onset of the experiment were tested by T cell proliferation assays.

AN-PEP accelerates the degradation of gluten present in white bread

After the introduction of the white bread to the TIM-system, the levels of all gluten peptides tested steadily decreased in the water-soluble compartment in the absence of AN-PEP. However, even after 120 minutes significant levels of gluten peptides were still detectable in the stomach compartment (Figure 2). In contrast, when the white bread was introduced together with AN-PEP the digestion of gliadins (Figure 2A and 2B) and glutenins (Figure 2C and 2D) was accelerated at all time points.

Samples that were collected from the duodenum, jejunum and ileum compartments contained very low levels of gluten peptides. In the case of digestion in the presence of AN-PEP no gluten peptides could be detected in these samples at all (results not shown).

Similarly, the Western blot analysis indicated that the degradation of gluten due to the addition of AN-PEP was accelerated. In the water-insoluble fractions of the stomach compartment α-gliadin, γ-gliadin and HMW-glutenin proteins were detectable (Figure 3). Although a significant degradation of gluten was seen in the absence of AN-PEP, both gliadins (Figure 3A and 3B) and glutenins (Figure 3C) were still present after 120 minutes in the stomach compartment. In the presence of AN-PEP, however, faster degradation of both the gliadins (Figure 3A and 3B) and the glutenins (Figure 3C) occurred. This difference in degradation is already apparent after 45 minutes, and after 90 minutes gluten proteins could no longer be detected in the AN-PEP treated fractions (Figure 3).

The duodenum fractions contained only a small amount of water-insoluble material. Western blot analysis failed to detect intact gluten proteins in these fractions, neither in the absence nor presence of AN-PEP (results not shown).

AN-PEP accelerates the degradation of gluten present in a complex meal

In the second experiment the digestion of gluten in a fast food menu was monitored. In the absence of AN-PEP, gluten peptides were found to be degraded in the water-soluble fraction. However, residual gluten peptides were still detectable in the fractions collected from all compartments up to 150 minutes after the start of the experiment (Figure 4). In the presence of AN-PEP, the degradation of gliadins (Figure 4B) and glutenins (Figure 4D) was accelerated. In the absence of AN-PEP, gluten peptides could be detected in the samples that were collected from the duodenum, jejunum and ileum compartments (Figure 4A and 4C). In the presence of AN-PEP much lower amounts of gluten peptides could be detected in these samples (Figure 4B and 4D).

Again, the analysis of the water-insoluble material collected from the stomach and duodenum compartment indicated accelerated degradation of the gluten in the meal. In the presence of AN-PEP the amount of α-gliadin is clearly decreased in the stomach compared to the digestion in the absence of AN-PEP (Figure 5A). Directly after homogenization (t = 0 min) a clear difference is apparent and after 60 minutes no more gluten proteins could be detected in the AN-PEP samples while such proteins were clearly still present in the control samples. Similarly, the HMW-glutenins were degraded faster in the presence of AN-PEP (Figure 5C). After 60 minutes no more HMW-glutenins could be detected in the AN-PEP fractions while partially degraded HMW-glutenins were still present in the control fractions (Figure 5C). Finally, the degradation of the γ-gliadins and LMW-glutenins was similarly enhanced by the addition of AN-PEP (Figure 5B and 5D).

Similar to the white bread experiment, the amount of water-insoluble material in the duodenal fractions was too small to allow detection of gluten proteins (result not shown).

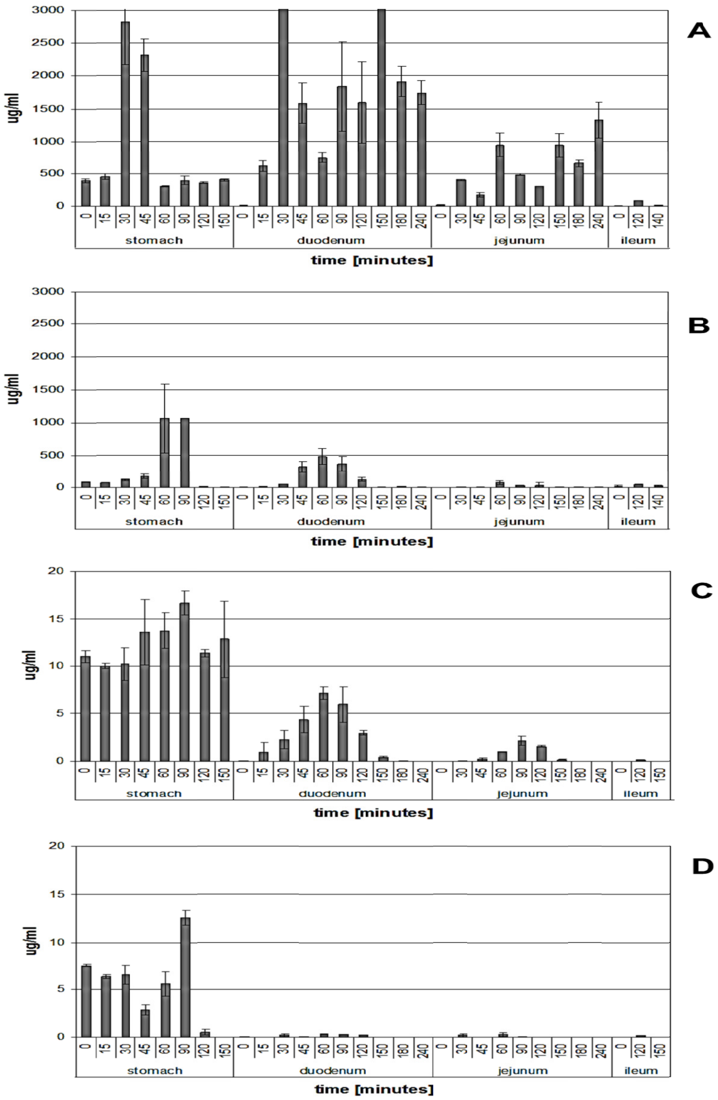

Figure 4. The presence of T cell stimulatory epitopes of α-gliadin and HMW-glutenin in the water-soluble fractions of a fast food menu digested in the TIM system.

A fast food menu, supplemented with additional bread (in total 50 g bread) was digested in the TIM system in the presence or absence of AN-PEP. Samples were taken at the indicated time points from the stomach, duodenum, jejunum and ileum compartments. The samples were divided in water-soluble and water-insoluble material and the level of T cell stimulatory epitopes in the water-soluble material was determined with mAb based competition assays. Levels of the Glia-α20 gliadin epitope (this study) in the absence (A) and presence (B) of AN-PEP. Levels of the HMW glutenin epitope (16) in the absence (C) and presence (D) of AN-PEP.

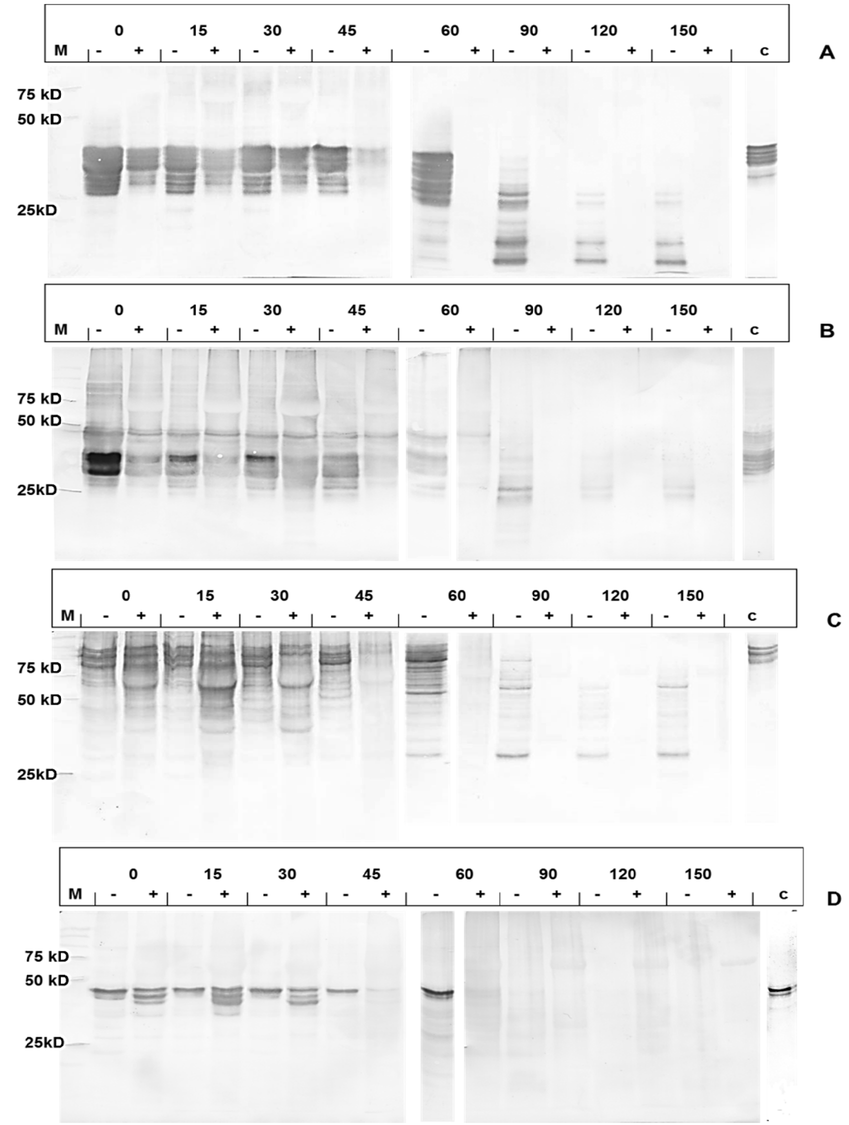

Figure 5. The presence of T cell stimulatory epitopes of α- and γ-gliadin, HMW- and LMW-glutenin in the water-insoluble fractions of a fast food menu digested in the TIM system.

A fast food menu, supplemented with additional bread (in total 50 g of bread) was digested in the TIM system in the presence (+) or absence (-) of AN-PEP. At the indicated time points samples were taken from the stomach compartment and the proteins present in the water-insoluble part of the samples were separated by 1D SDS-PAGE. After transfer to a PVDF membrane the presence of proteins containing T cell stimulatory epitopes of Glia-α20 (A), Glia-γ1 (B), HMW-glutenin (C) and LMW- glutenin (D) were visualized using epitope specific mAb (16;32) (this study). In this figure c is a gluten control.

Small amounts of gluten peptides could still be detected in the AN-PEP treated samples that were measured with the antibody based competition assays. As these assays detect gluten fragments that are smaller than those required for T cell stimulation, we determined if AN-PEP destroyed the T cell stimulatory properties in the TIM-system. T cell clones specific for the Glia-α9, Glia-α20, LMW glutenin and the HLA-DQ8 restricted HMW glutenin derived T cell epitopes were incubated with the untreated and the AN-PEP treated samples and T cell proliferation was measured (Figure 6). While in the untreated samples T cells stimulatory activity was clearly present, AN-PEP treatment completely abolished this activity.

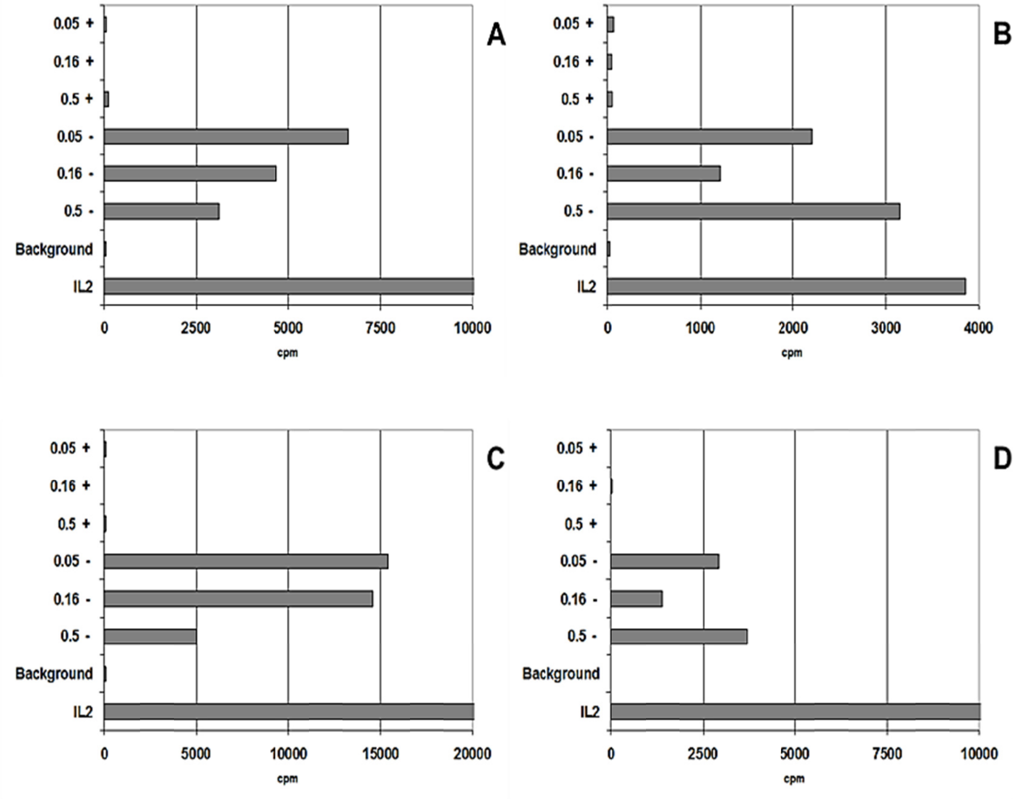

Figure 6. Stimulation of gluten specific T cells by samples of the fast food menu collected at 60 minutes after the addition of AN-PEP from the stomach compartment of the TIM system.

Three different concentrations of the stomach samples: 0.5 µl, 0.16 µl and 0.05 respectively collected from the experiment of the fast food digestion in the presence (+) or absence (-) of AN-PEP were tested in T cell proliferation assays. Stimulation of T cell clones recognizing the Glia-α9 epitope (A), Glia-α20 epitope (B), the HLA DQ8 molecule restricted HMW-Glt epitope (C) and LMW Glt-156 epitope (D) is shown.

DISCUSSION

It is well established that CD patients are intolerant to gluten and that a strict gluten-free diet (GFD) is an effective treatment for CD. The lifelong maintenance of such a diet, however, is hard to achieve. First of all, the diet in the western world is heavily based on gluten containing foods like bread and pasta. In addition, because of its special properties, like its elasticity and capacity to bind water, gluten is often added to foods that would otherwise be gluten-free. Finally, many foods that are rendered gluten-free may still contain traces of gluten while naturally gluten-free ingredients are often contaminated with gluten. CD patients, therefore, can inadvertently be exposed to gluten. Moreover, a GFD can cause social constraints and this can lead to non-compliance with the diet. For these reasons an alternative to a GFD would be useful to patients. Oral supplementation with enzymes to degrade gluten before it causes damage has been suggested for this purpose. Initially this proposition was based on the theory that CD is caused by an enzyme deficiency (34) and the use of non-human proteases for gluten detoxification was already proposed in the late fifties (35). Recently a clinical trial was performed in which an extract from animal intestines was used. It was shown that the enzyme therapy offered better protection than placebo. However, the differences between enzyme therapy and placebo were small and no complete protection was given by the extract. Furthermore, not all patients did benefit to the same extent from the enzyme supplementation (36).

As gluten is rich in the amino acid proline, other recent studies have used post-proline cutting enzymes. Promising enzymes tested are the prolyl oligopeptidases from *Flavobacterium meningosepticum, Sphingomonas capsulate*, and *Myxococcus xanthus*. These enzymes are capable of degrading proline containing peptides that are otherwise resistant to degradation by proteases in the gastrointestinal tract *in vitro* (23;37;38). However, the suitability of these enzymes to degrade gluten *in vivo* is questionable since the enzymes have a pH optimum between 7 and 8 and do not function at the acid pH of the stomach. Moreover, they are efficiently broken down by pepsin (23). Finally, due to their structure, in which a β-propeller domain restricts entry into the active site of the enzymes, they preferentially cleave short peptides (39). Encapsulation of these prolyl oligopeptidases was proposed to protect them against gastric juice (38). However, in a recent *ex vivo* study, using biopsy-derived intestinal tissue mounted in Ussing chambers, it was observed that only high dosages of prolyl oligopeptidase tested were capable to eliminate the accumulation of immunogenic peptides in the serosal compartment (21). This indicates that, even if the enzyme is encapsulated, due to the relatively low efficiency the prolyl oligopeptidase will not be able to degrade gluten before it reaches the proximal part of the duodenum, the site where gluten triggers inflammatory T cell responses. To overcome these problems a combination therapy has been proposed. For this the prolyl oligopeptidase is combined with a cysteine endoprotease from barley whose natural function is to degrade the gluten-like molecules in barley, to the benefit of the germinating seed. While such a combination therapy may prove effective, a therapy based on one enzyme would have obvious advantages (40).

Recently, we have investigated a prolyl endoprotease of *Aspergillus niger,* AN-PEP (31). We observed that AN-PEP has clear advantages over prolyl oligopeptidases as it is much more efficient in degrading gluten peptides, is active at low pH, and resistant to pepsin degradation (31;41). Moreover, as AN-PEP is a prolyl endoprotease, it degrades gluten peptides as well as intact gluten proteins (31;41).

These results indicated that AN-PEP may be suitable as an oral supplement for gluten degradation but its efficacy *in vivo* remained to be established. Although this can be tested in animal models, as has been carried out for a prolyl oligopeptidase, the relevance of this for the human situation is debatable as the gastrointestinal tract of animals only partly reflects that of humans. We have therefore chosen to study the efficacy of AN-PEP using a dynamic gastrointestinal model that closely mimics the *in vivo* conditions found in the human stomach and small intestine (26-28). Moreover, we have tested the capacity of AN-PEP to degrade gluten when present in a relatively simple food matrix, e.g. white bread, as well as when gluten is present in complex food matrix, e.g. a complete fast food meal. The results of this study show that AN-PEP is indeed capable of degrading gluten under conditions found in the human GI-tract. We have carried out three types of analysis: competition assays that measure the presence of small gluten fragments in the water-soluble fractions, Western blot to measure intact gluten proteins and relatively large fragment thereof (> 5 kD) and T cell proliferation experiments that measure the presence of gluten derived T cell stimulatory peptides. Both the competition assays and the Western blot analysis gave comparable results: the disappearance of gluten proteins as indicated by Western blot analysis also led to a disappearance of smaller gluten peptides as measured in the competition assay. Moreover T cell proliferation assays on samples collected at 60 minutes from the digesting fast food menu showed that after the addition of the AN-PEP, the stomach content is no longer capable to induce a T cell proliferation. Taken together, these results indicate that AN-PEP completely degrades gluten into harmless fragments within 2 hours, the average passage time of food in the stomach. Besides, during the stay of the gluten in the stomach, the level of T cell stimulatory epitopes of α- and γ-gliadin HMW-glutenin and LMW-glutenin epitopes in the water soluble fragments were significantly lower in the presence of AN-PEP than in the absence. This results in much lower amounts of T cell stimulatory epitopes that are released from the stomach into the duodenum, thereby reducing the exposure of the proximal duodenum with T cell activating peptides. Moreover, in a previous study we have already demonstrated that AN-PEP will efficiently cut the alpha-gliadin peptide 31-43 and thus destroy the innate stimulatory properties of gluten.

It is important to note that our results may underestimate the rate of gluten degradation in the human body. Although the TIM-system simulates the lumen of the human GI-tract, including peristaltic movements, secretion of juices from the salivary gland, the stomach, pancreas and liver, no brush border enzymes are present in the model. As brush border enzymes have an additive effect on proteolysis, which is also shown for the breakdown of gluten proteins (25), it is likely that the degradation of gluten is even more efficient than indicated by our results.

In conclusion, we have tested the effect of the addition of AN-PEP on gluten degradation in a system that closely mimics the conditions in the upper human GI-tract. Our results demonstrate that within the time span that food is normally present in the stomach, co-administration of AN-PEP led to a complete disappearance of T cell stimulatory peptides of gliadins and glutenins. Importantly, our results demonstrate that AN-PEP is capable of degrading gluten when this is present in a complex food matrix. Moreover, AN-PEP is derived from the food grade microorganism *Aspergillus niger* and available on industrial scale. Ultimately, clinical trials will be required to determine if oral enzyme supplementation can remove all gluten toxicity. Our results indicate that AN-PEP is a very suitable candidate for testing in such trials.

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COMPETING INTERESTS

L. Edens is an employee of the DSM Food Specialties. Part of the work presented here has been patented by DSM.

REFERENCES

1. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992;**102**(1):330-54.
2. Shewry PR. Plant storage proteins. *Biol Rev Camb Philos Soc* 1995;**70**(3):375-426.
3. Molberg O, McAdam SN, Korner R *et al*. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat Med* 1998;**4**(6):713-7.
4. Kwok WW, Domeier ML, Raymond FC *et al*. Allele-specific motifs characterize HLA-DQ interactions with a diabetes- associated peptide derived from glutamic acid decarboxylase. *J Immunol* 1996;**156**(6):2171-7.
5. van de Wal Y, Kooy YMC, Drijfhout JW *et al*. Peptide binding characteristics of the coeliac disease-associated DQ(alpha1*0501, beta1*0201) molecule. *Immunogenetics* 1996;**44**(4):246-53.
6. Vartdal F, Johansen BH, Friede T *et al*. The peptide binding motif of the disease associated HLA-DQ (alpha 1* 0501, beta 1* 0201) molecule. *Eur J Immunol* 1996;**26**(11):2764-72.
7. Vader LW, De Ru A, van der WY *et al*. Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. *J Exp Med* 2002;**195**(5):643-9.
8. Vader W, Kooy Y, van Veelen P *et al*. The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. *Gastroenterology* 2002;**122**(7):1729-37.
9. van de Wal Y, Kooy YM, van Veelen PA *et al*. Small intestinal T cells of celiac disease patients recognize a natural pepsin fragment of gliadin. *Proc Natl Acad Sci U S A* 1998;**95**(17):10050-4.
10. Anderson RP, Degano P, Godkin AJ *et al*. 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**(3):337-42.
11. 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**(4):603-12.
12. Arentz-Hansen H, McAdam SN, Molberg O *et al*. Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. *Gastroenterology* 2002;**123**(3):803-9.
13. Sjostrom H, Lundin KE, Molberg O *et al*. Identification of a gliadin T-cell epitope in coeliac disease: general importance of gliadin deamidation for intestinal T-cell recognition. *Scand J Immunol* 1998;**48**(2):111-5.
14. van de Wal Y, Kooy YM, van Veelen P *et al*. Glutenin is involved in the gluten-driven mucosal T cell response. *Eur J Immunol* 1999;**29**(10):3133-9.
15. Molberg O, Solheim FN, Jensen T *et al*. Intestinal T-cell responses to high-molecular-weight glutenins in celiac disease. *Gastroenterology* 2003;**125**(2):337-44.

16. Spaenij-Dekking L, Kooy-Winkelaar EM, van Veelen PA *et al*. Natural variation in toxicity of wheat: potential for selection of non-toxic varieties for coeliac disease patients. *Gastroenterology* 2005;(129):797-806.

17. Spaenij-Dekking L, Kooy-Winkelaar EM, Koning F. The Ethiopian Cereal Tef in Celiac disease. *N Engl J Med* 2005;**353**(16):1748-9.

18. Molberg O, Uhlen AK, Jensen T *et al*. Mapping of gluten T-cell epitopes in the bread wheat ancestors: implications for celiac disease. *Gastroenterology* 2005;**128**(2):393-401.

19. Shan L, Qiao SW, Arentz-Hansen H *et al*. Identification and analysis of multivalent proteolytically resistant peptides from gluten: implications for celiac sprue. *J Proteome Res* 2005;**4**(5):1732-41.

20. Pyle GG, Paaso B, Anderson BE *et al*. Effect of pretreatment of food gluten with prolyl endopeptidase on gluten-induced malabsorption in celiac sprue. *Clin Gastroenterol Hepatol* 2005;**3**(7):687-94.

21. 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**(3):786-96.

22. Marti T, Molberg O, Li Q *et al*. Prolyl endopeptidase-mediated destruction of T cell epitopes in whole gluten: chemical and immunological characterization. *J Pharmacol Exp Ther* 2005;**312**(1):19-26.

23. Shan L, Marti T, Sollid LM *et al*. Comparative biochemical analysis of three bacterial prolyl endopeptidases: implications for coeliac sprue. *Biochem J* 2004;**383**(Pt 2):311-8.

24. Piper JL, Gray GM, Khosla C. Effect of prolyl endopeptidase on digestive-resistant gliadin peptides in vivo. *J Pharmacol Exp Ther* 2004.

25. Hausch F, Shan L, Santiago NA *et al*. Intestinal digestive resistance of immunodominant gliadin peptides. *Am J Physiol Gastrointest Liver Physiol* 2002;**283**(4):G996-G1003.

26. Minekus M, Marteau P, Havenaar R *et al*. A multicompartmental dynamic computer controlled model simulating the stomach and small intestine. *Alternatives to Laboratory Animals (ATLA)* 1995;**23**:197-209.

27. Blanquet S, Zeijdner E, Beyssac E *et al*. A dynamic artificial gastrointestinal system for studying the behavior of orally administered drug dosage forms under various physiological conditions. *Pharm Res* 2004;**21**(4):585-91.

28. Souliman S, Blanquet S, Beyssac E *et al*. A level A in vitro/in vivo correlation in fasted and fed states using different methods: applied to solid immediate release oral dosage form. *Eur J Pharm Sci* 2006;**27**(1):72-9.

29. Verwei M, Arkbage K, Havenaar R *et al*. Folic acid and 5-methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model. *J Nutr* 2003;**133**(7):2377-83.

30. Salovaara S, Larsson AM, Eklund-Jonsson C *et al*. Prolonged transit time through the stomach and small intestine improves iron dialyzability and uptake in vitro. *J Agric Food Chem* 2003;**51**(17):5131-6.

31. Edens L, Dekker P, van der HR *et al*. Extracellular prolyl endoprotease from Aspergillus niger and its use in the debittering of protein hydrolysates. *J Agric Food Chem* 2005;**53**(20):7950-7.

32. Spaenij-Dekking EH, Kooy-Winkelaar EM, Nieuwenhuizen WF *et al*. A novel and sensitive method for the detection of T cell stimulatory epitopes of alpha/beta- and gamma-gliadin. *Gut* 2004;**53**(9):1267-73.
33. Eckert R van. The PWG gliadin, a new refernce material. Proceedings of the 16th meeting Working Group on Prolamin Analysis and Toxicity. Sitges, Spain . 2000.
34. FRAZER AC, FLETCHER RF, ROSS CA *et al*. Gluten-induced enteropathy: the effect of partially digested gluten. *Lancet* 1959;**2**:252-5.
35. KRAINICK HG, MOHN G. [Further investigations on the toxic effect of wheat flour in celiac disease. 2. Effect of enzymatic by-products of gliadin.]. *Helv Paediatr Acta* 1959;**14**(2):124-40.
36. Cornell HJ, Macrae FA, Melny J *et al*. Enzyme therapy for management of coeliac disease. *Scand J Gastroenterol* 2005;**40**(11):1304-12.
37. Shan L, Molberg O, Parrot I *et al*. Structural basis for gluten intolerance in celiac sprue. *Science* 2002;**297**(5590):2275-9.
38. Gass J, Ehren J, Strohmeier G *et al*. Fermentation, purification, formulation, and pharmacological evaluation of a prolyl endopeptidase from Myxococcus xanthus: implications for Celiac Sprue therapy. *Biotechnol Bioeng* 2005;**92**(6):674-84.
39. Polgar L. Prolyl endopeptidase catalysis. A physical rather than a chemical step is rate-limiting. *Biochem J* 1992;**283 (Pt 3)**:647-8.
40. Siegel M, Bethune MT, Gass J *et al*. Rational design of combination enzyme therapy for celiac sprue. *Chem Biol* 2006;**13**(6):649-58.
41. 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.