Celiac disease : from basic insight to therapy development

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Am J Physiol Gastrointest Liver Physiol.
2006 Oct;291(4):G621-9.

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ABSTRACT

Celiac disease is a T cell-driven intolerance to wheat gluten. The gluten derived T cell epitopes are proline-rich and thereby highly resistant to proteolytic degradation within the gastrointestinal tract. Oral supplementation with prolyl oligopeptidases has therefore been proposed as a potential therapeutic approach. The enzymes studied, however, have limitations as they are irreversibly inactivated by pepsin and acidic pH, both present in the stomach. As a consequence, these enzymes will fail to degrade gluten before it reaches the small intestine, the site where gluten induces inflammatory T cell responses that lead to celiac disease. We have now determined the usefulness of a newly identified prolyl endoprotease from *Aspergillus niger* for this purpose. Gluten and its peptic/tryptic digest were treated with prolyl endoprotease and the destruction of the T cell epitopes was tested using mass spectrometry, T cell proliferation assays, ELISA, rpHPLC, SDS-PAGE and Western blotting. We observed that the *A. niger* prolyl endoprotease works optimally at pH 4-5, remains stable at pH 2 and is completely resistant to digestion with pepsin. Moreover, the *A. niger* derived enzyme efficiently degraded all tested T cell stimulatory peptides as well as intact gluten molecules. On average the endoprotease from *A. niger* degraded gluten peptides 60 times faster than a prolyl oligopeptidase. Together these results indicate that the enzyme from *A. niger* efficiently degrades gluten proteins. Future studies are required to determine if the prolyl endoprotease can be used as an oral supplement to reduce gluten intake in patients.

INTRODUCTION

Celiac disease is a chronic enteropathy caused by an uncontrolled immune response to wheat gluten and similar proteins of rye and barley. Upon ingestion, proteases in the gastrointestinal tract degrade gluten proteins into peptides. The enzyme tissue transglutaminase modifies these peptides, by deamidating glutamine residues into glutamic acid [1,2,3]. Subsequently, these peptides bind to either HLA-DQ2 or HLA-DQ8 molecules and evoke T cell responses leading to inflammation in the small intestine and ultimately to the typical symptoms associated with celiac disease: diarrhoea, malnutrition and failure to thrive.

A peculiar feature of the T cell stimulating peptides is their high proline content. Proline constitutes 12-17% of wheat gluten and the gluten-like molecules in barley and rye contain similar amounts [4]. Since human gastric, and pancreatic enzymes lack post-proline cleaving activity the abundance of proline residues in gluten renders it highly resistant to complete proteolytic degradation in the human gastrointestinal tract, a feature that is most likely linked to the disease inducing properties of gluten.

The use of non-human proteases for gluten detoxification was already proposed in the late nineteen fifties [5] and a clinical trial took place in 1976 [6] but did not provide clear-cut conclusions. Recently, it has been shown that prolyl oligopeptidase from *Flavobacterium meningosepticum* (FM-POP) is capable of breaking down toxic gluten sequences in vitro [7]. Prolyl oligopeptidases from *Sphingomonas capsulate* and *Myxococcus xanthus* were also studied and have comparable properties [8,9]. Prolyl oligopeptidases, however, have pH optima between 7 and 8 so that they cannot function at the acid pH in the stomach. Also, they are efficiently broken down by pepsin [8]. Besides, due to their structure, in which a β-propeller domain restricts entry into the active center, the enzymes preferentially cleave short peptides [10]. These properties imply that oral supplementation with prolyl oligopeptidases will not be sufficient to degrade gluten before it reaches the proximal parts of the duodenum, which is in agreement with observations published recently by Matysiak-Budnik et al. [11].

In the present study we have investigated a newly discovered prolyl endoprotease from *Aspergillus niger* (AN-PEP) [12]. AN-PEP is a member of the serine peptidase family S28 and shares more sequence homology with lysosomal Pro-X carboxypeptidase and dipeptidyl peptidase II than with prolyl oligo-peptidases. The results of the present study indicate that AN-PEP efficiently degrades gluten in vitro under the conditions similar to the ones present in the gastrointestinal tract. Since no animal model for celiac disease is available an in vivo evaluation of the efficacy of AN-PEP will ultimately have to be carried out in patients. As large amounts of pure

AN-PEP can be produced relatively cheaply, efficiently and at food grade quality the enzyme appears a good candidate for such studies.

MATERIALS AND METHODS

Reagents

Pepsin (2331 u/mg), trypsin (9600 u/mg), chymotrypsin (54 u/mg), guinea pig tissue transglutaminase (1.68 u/mg), pepstatin A, phenylmethylsulfonyl fluoride (PMSF) and standard 4-nitroaniline (pNA) were from Sigma (St. Louis, MO). Prolyl oligopeptidase from *Flavobacterium meningosepticum* (FM-POP, 35U/mg) was from ICN Biochemicals Inc. (Aurora, Ohio). Prolyl endoprotease from *Aspergillus niger* (AN-PEP) was produced and purified by DSM Food Specialties (Delft, The Netherlands). Besides post proline cleaving activity no other exo- or endoproteolytic activities were detected in the preparation. N-carbobenzyloxy-glycyl-proline-4-methyl-7-coumarinylamide (Z-Gly-Pro-AMC) and standard 4-methyl-7-coumarinyl-amide (AMC) were from Fluka Chemie AG (Buchs, Switzerland). Acetyl-alanine-alanine-proline-4-nitroaniline (Ac-Ala-Ala-Pro-pNA) was produced in our own peptide synthesis facility. Protein concentrations were determined using a Bradford protein assay kit (Bio Rad, Munchen, Germany).

pH optimum

The pH optimum of AN-PEP and FM-POP was determined using 200 μM Z-Gly-Pro-AMC as a substrate, which was prepared in a range of 100 mM buffers at various pH values. The buffers used were citric acid/ NaOH (pH 2-6), Tris/HCl (pH 6-8) and glycine/NaOH (pH 8-12). The concentration of AN-PEP and FM-POP was 32 μg/ml and 0.2 μg/ml respectively. The reaction was carried out for 30 minutes at 37°C. The released AMC was measured fluorimetrically at λ_{ex} 360 nm and λ_{em} 460 nm using a CytoFluor multi-well plate reader (PerSeptive Biosytems, Framingham, MA).

Stability at low pH and resistance to pepsin degradation

Both AN-PEP and FM-POP were diluted with 100 mM glycine/HCl buffer pH 2.0 to 1 mg/ml and mixed with an equal volume of 100 μM (i.e. 3.5 mg/ml) pepsin in the same buffer and incubated at 37°C. At the time points of 0, 15, 30 and 60 minutes 80 μl samples were taken and transferred into tubes containing 2 μl of 0.8 mM pepstatin and immediately frozen and stored at –20°C until measurements of enzymatic activity.

Activity assays

The activity of FM-POP was measured using the fluorogenic substrate Z-Gly-Pro-AMC. The assay was performed in 96-well black plates with a clear bottom (Corning Inc., NY, USA). Every measurement was performed in duplicate. The enzyme samples were diluted in 100mM Tris/HCl buffer pH 7.0 to a final concentration of 0.1 µl/ml. The reaction was started by mixing 95 µl enzyme with 5 µl of substrate (4 mM in 60% methanol). After 30 minutes at 37°C, the reaction was stopped with 50µl of 1M acetic acid. The released AMC was measured as described above. The activity of AN-PEP was determined using the substrate Ac-Ala-Ala-Pro-pNA. The assay was performed in 96-well transparent plates. Every measurement was performed in duplicate. The enzyme samples were diluted in 100 mM sodium acetate buffer pH 4.5 to a final concentration of 0.1 µg/ml. The reaction was started by mixing 50 µl enzyme with 50 µl substrate (400 µM in 100 mM sodium acetate buffer pH 4.5). After 30 minutes at 37°C the absorption at 405 nm was measured using an ELISA plate reader (Spectro Classic, Wallac).

Enzymatic digestions and mass spectrometry

Synthetic peptides were dissolved in water at a concentration of 1mg/ml and mixed with an equal volume of FM-POP solution in 50 mM ammonium acetate buffer pH 7.0 or AN-PEP in 50 mM ammonium acetate buffer pH 4.5. The final concentration of FM-POP in the reaction was 10 µg/ml and the final concentration of AN-PEP was 0.5 µg/ml. At time points 15, 30, 60 and 120 minutes 0.5 µl aliquots of the reaction mixture were taken and mixed with 9.5 µl of matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile with 0.2 % trifluoroacetic acid), directly loaded on a MALDI-TOF-MS plate and dried at room temperature. The mass spectra were obtained with a Voyager DE™ PRO instrument (PerSeptive Bio-systems, Framingham, MA) in linear ion mode. The cleavage sites of the peptides were calculated using MassLynx software as supplied with the Q-TOF1 (Micromass, Manchester, England). Selected peptides were sequenced using electrospray ionisation mass spectrometry on a Q-TOF1 as described [1].

Degradation rate measurements

Synthetic peptides were dissolved in water at a concentration of 4 mg/ml. The AN-PEP enzyme was diluted in 100 mM citrate buffer pH 4.5. FM-POP was dissolved in 100 mM sodium acetate buffer pH 7.0. The concentration of both enzymes was 2 µg/ml. The reaction was started by mixing the peptide and enzyme solutions at equal volumes. At time points of 2, 5, 10, 30, 60 and 120 minutes 40 µl aliquots were removed from the reaction mixture and added to either 40 µl 1M ammonia (in case of AN-PEP digestions) or 10 mM PMSF in water was added (in case of FM-POP digestions). Control samples were prepared by mixing peptide with inactivated

enzyme. All samples were diluted in water, acetonitrile, and acetic acid in a v/v/v ratio of 95:3:1 to a concentration of 2 μg/ml. A standard peptide was added to a final concentration of 0.4 μg/ml. Spectra of the samples were obtained with a Q-TOF1 mass spectrometer. The ratio of the peaks of the standard and target peptide was calculated and expressed as a percentage value.

AN-PEP treatment of peptic/tryptic gluten digest

The gliadin peptic/tryptic digest was prepared as described [13]. The obtained preparation was deamidated with guinea pig tissue transglutaminase (100 μg/ml; Sigma) in PBS with 2 mM $CaCl_2$ overnight at 37°C. Subsequently the pH was adjusted to 4.5 with HCl and the digest (0.7 mg/ml) was treated with AN-PEP (3.5 μg/ml) for 120 minutes at 37°C in a total volume of 520 μl. The reaction was stopped by the addition of 5 μl of 1M NaOH after which the pH was adjusted to 7. Control samples were prepared by mixing the peptic/tryptic digests with inactivated enzyme. The degradation of gluten T cell stimulatory epitopes was monitored with T cell proliferation assay as described below.

For the competition assays with antibodies specific for the T cell stimulatory epitopes a peptic/typtic digest of gluten was treated with AN-PEP in the following way: 2 ml of gluten peptic/tryptic digest (4mg/ml) were mixed 2 ml of 100 mM citrate buffer, pH 4.5 and incubated for 5 minutes at 37°C. Subsequently 40 μl of AN-PEP (1mg/ml) was added and at time points 5, 10, 15, 30, 60, 120 minutes and 20 hours the digestion was stopped by transferring 400 μl aliquots of the samples to vials containing 10 μl 10 M NaOH after which the pH was adjusted to 7. Control samples were prepared by mixing the peptic/tryptic digest with inactivated enzyme. After adjusting pH of the samples to about 7 the content of α- and γ-gliadins as well as high and low molecular weight glutenins was determined.

Digestion of whole gluten – protocol 1

1 g of gluten (Sigma, St. Louis, MO) was suspended in 20 ml 1 M acetic acid and ultrasonicated for 30 minutes. Insoluble material was removed by centrifugation for 15 min. at 3000 rpm. Subsequently the pH of the supernatant was adjusted to 4.5 with ammonia and the resulting precipitate removed by centrifugation for 15 min at 3000 rpm. Protein concentration in the supernatant was determined with a Bradford assay and adjusted to 1mg/ml by the addition of 100 mM citrate buffer pH 4.5. Subsequently 5 μl of AN-PEP (1mg/ml) was added to 1 ml of the gluten solution and incubated for 2 hours at 37°C after which 100 μl of the sample was mixed with 300 μl 10 % acetic acid and, after removal of insoluble material by centrifugation for 5 min at 14.000 rpm, separated by rpHPLC on a C2/C18 sc 2.1/10 column (Smart, Pharmacia, Uppsala, Sweden). As controls the samples of undigested gluten and

AN-PEP were separated in an identical fashion. Collected fractions were subsequently analyzed with a Q-TOF1 mass spectrometer (Micromass, Manchester, England). Individual peptides were sequenced with an ion trap mass spectrometer (HCTplus, Bruker Daltonics, Bremen, Germany). Several fractions collected at a retention time of 25 to 42 minutes were applied to15% SDS-PAGE under reducing conditions. The proteins were either visualized with Coomassie Brillant Blue (Imperial Protein Stain, Pierce, Rockford, IL, USA) or transferred to nitrocellulose for subsequent Western blotting with monoclonal antibodies specific for α- and γ-gliadin, and HMW- and LMW-glutenin as described [14,15]

Digestion of whole gluten – protocol 2

2 g of gluten (Sigma, St. Louis, MO) was suspended in 100 ml of 10 mM HCl and the pH was adjusted to 4.5 with NaOH. During the entire experiment gentle stirring with a magnetic stirrer was applied. The digestion was initiated by the addition of 30 mg of pepsin and 20 mg of AN-PEP to the gluten suspension. After 1-hour incubation at 37°C the pH was adjusted to 2.0 with HCl, additional 30 mg of pepsin was added and the suspension was incubated for the next hour. Thereafter the pH was adjusted to 7.9 with NaOH and trypsin (20 mg) and chymotrypsin (20 mg) were added. This was incubated for 1 hour at 37°C and boiled for 10 minutes to inactivate the enzymes. Similarly, the controls with only pepsin, pepsin/AN-PEP and pepsin/trypsin/chymotrypsin were prepared. The samples were frozen and stored at -80°C until further tested by western blotting, competition assays and T cell proliferation tests.

Western blotting

To determine the level of T cell stimulatory epitopes present in the gluten digests, the digest samples 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) and run on a 12,5% SDS-PAGE gels. The proteins were visualized either directly using Imperial Protein Stain (Pierce, Rockford IL, USA), or after transfer to nitrocellulose membranes with the mAbs specific for stimulatory T cell epitopes from α- and γ-gliadin and HMW- and LMW-glutenins [14,15].

Competition assay

After adjusting the pH of the samples to about 7 the content of α- and γ-gliadins as well as HMW- and LMW-glutenins was determined using competition assays specific for T cell stimulatory epitopes involved in celiac disease as described previously [14,15]. Briefly, microtiter plates (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 (RT). Plates were washed in PBS/ 0,02% Tween-20 and residual binding sites were blocked with PBS 1% Skim milk powder (Fluka, the Netherlands). Of the gluten containing samples different dilutions were made in 20 mM NaH_2PO_4/ Na_2HPO_4 pH 7.0/ 0.1% Tween-20/ 0.1% skim milk and these were mixed with either a biotinylated α- or γ- gliadin T cell epitope encoding peptides. The mixtures were incubated on the plates for 1.5 h at RT. Next plates were washed and incubated for 30 min with streptavidin conjugated horseradish peroxidase in PBS/ 0.1% skim milk, hereafter bound peroxidase was visualized as described. For quantification of the gliadin assays a standard curve was made with the European gliadin reference IRMM-480 in a concentration range of 10 μg/ml-10 ng/ml. For the LMW-glutenin assay a standard curve was made using the synthetic peptide QPPFSQQQQPPFSQQQQSPFSQQQQ-amine in a concentration range from 1 μg/ml –1 ng/ml. For the HMW-glutenin assay a standard curve was made using a trypsin/chymotrypsin digest of recombinant HMW-glutenin proteins (kindly provided by P. Shewry, Rothamsted Research, Hampenden, United Kingdom) in a concentration range from 1 μg/ml-1 ng/ml. The assays were repeated at least twice.

T-cell proliferation assay

The gluten digest samples were thawn, centrifuged for 10 min at 18000g and incubated with guinea pig tissue transglutaminase (200μg/ml) and $CaCl_2$ (10 mM) for 1 hour at 37°C. Proliferation assays were performed in triplicate in 150 μl RPMI-1640 (Gibco) supplemented with 10% human serum in 96-well flat-bottom plates (Falcon) using 10^4 gluten specific T cells stimulated with 10^5 irradiated HLA-DQ2-matched allogeneic PBMCs (3000 RAD) in the presence of 15 μl of the gluten digests, an amount that had been shown not to be toxic to the T cells. After 48 hours at 37°C, cultures were pulsed with 0.5 μCi of ^{3}H-thymidine, harvested 18 hours later and the thymidine incorporation was quatified with a liquid scintillation counter. Culture flasks and other disposables were from Greiner (Frickenhausen, Germany).

RESULTS

AN-PEP is active at pH present in the stomach

To determine the pH optimum of AN-PEP, the enzyme was incubated with a fluorogenic substrate Z-Gly-Pro-AMC in buffers spanning the pH range of 2-12 and the activity measured (Fig. 1). AN-PEP activity was detected at pH 2-8, with optimum between pH 4 and pH 5. In contrast, the pH optimum of FM-POP is about pH 7-8 and the enzyme is not active at pH below 5.

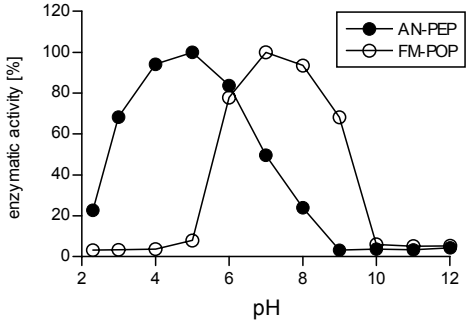


Figure 1. Comparison of the pH optima of AN-PEP and FM-POP. The hydrolytic activity of the enzymes was measured fluorimetrically with the fluorogenic substrate Z-Gly-Pro-AMC.

AN-PEP is resistant to low pH and digestion by pepsin

To compare the resistance of FM-POP and AN-PEP to the conditions present in the stomach the enzymes were incubated at pH 2.0 in the presence or absence of pepsin (1.75 mg/ml). After 0, 15, 30 and 60 minutes the pepsin was inactivated by the addition of the inhibitor pepstatin A and the remaining enzyme activity in the samples was determined at the pH optima of the enzymes (Fig. 2). The results demonstrate that AN-PEP was entirely resistant to incubation at pH 2.0 and degradation by pepsin. In contrast, incubation of FM-POP for 15 minutes at pH 2.0 reduced its activity by approximately 50% while the combination of pH 2.0 and pepsin immediately inactivated FM-POP.

The AN-PEP enzyme degrades all tested gluten peptides

An effective enzymatic treatment for celiac disease requires means of destroying all or at least the vast majority of gluten derived T cell stimulatory sequences. To test whether AN-PEP meets this criterion the cleavage sites in a large number of gluten epitopes were determined (Table 1). In every T cell stimulatory epitope tested at least one major cleavage site of AN-PEP was present. Also the peptide Glia p31-49, known to stimulate innate responses in celiac patients, was efficiently proteolysed (Table 3). In general peptide bonds located in the middle of a peptide were more efficiently cleaved than those located near the N or C terminus. Due to the activity of the enzyme tissue transglutaminase glutamine residues in gluten peptides are frequently modified into glutamic acid in the small intestine. This modification, however, had no significant influence on AN-PEP activity and specificity (Table 2).

The rate of peptide degradation

Ingested food remains in the stomach usually between 1 and 4 hours. It is crucial that most of the toxic gluten sequences are destroyed before reaching the duodenum

Table 1. The most prominent cleavage sites of AN-PEP and FM-POP in selected T cell stimulatory gluten peptides. The peptides were treated with AN-PEP or FM-POP (at the pH optima of the enzymes) and the generated peptide fragments were identified by MALDI-TOF-MS. Minimal T cell stimulatory sequences are given in bold.

Epitope	Enzyme	Major cleavage sites
Glia 31-43	AN-PEP	**L G Q Q Q P**↓**F P P Q Q P**↓**Y** P↓Q P Q P F
	FM-POP	**L G Q Q Q P**↓**F P P**↓**Q Q P**↓**Y** P↓Q P Q P↓F
Glia-α2	AN-PEP	**P**↓**Q P Q L P**↓**Y P Q** P Q L P Y
	FM-POP	**P Q P Q L P**↓**Y P**↓**Q** P Q L P↓Y
Glia-α9	AN-PEP	Q L Q **P**↓**F P**↓**Q P Q L P**↓**Y**
	FM-POP	Q L Q **P**↓**F P**↓**Q P Q L P**↓**Y**
Glia-α20	AN-PEP	P **F R P**↓**Q Q P**↓**Y P**†**Q** P Q P Q
	FM-POP	P **F R P Q Q P**↓**Y P**↓**Q** P Q P†Q
Glia-γ1	AN-PEP	Q P Q Q **P**↓**Q Q S F P**↓**Q Q Q** R P↓F
	FM-POP	Q P Q Q **P Q Q S F P**↓**Q Q Q** R P↓F
Glia-γ2	AN-PEP	Q Q **P**↓**Y P Q Q P**↓**Q Q P F** P Q
	FM-POP	Q Q **P**↓**Y P**↓**Q Q P Q Q P**↓**F** P↓Q
Glia-γ30	AN-PEP	V Q G Q G **I I Q P**↓**Q Q P A Q** L
	FM-POP	V Q G Q G **I I Q P**↓**Q Q P**↓**A Q** L
Glt-17	AN-PEP	Q Q P **P**↓**F S Q Q Q Q Q P**↓L P Q
	FM-POP	Q Q P **P**↓**F S Q Q Q Q Q P**↓L P↓Q
Glt-156	AN-PEP	Q Q P **P**↓**F S Q Q Q Q S P**†**F** S Q
	FM-POP	Q Q P **P F S Q Q Q Q S P**↓**F** S Q
Glu-5	AN-PEP	Q Q U S Q P↓Q U P↓Q Q Q.**Q U P**↓**Q Q P Q Q F**
	FM-POP	Q Q U S Q P Q U P↓Q Q Q **Q U P**↓**Q Q P**↓**Q Q F**
Glu-21	AN-PEP	Q P Q P↓F P↓Q **Q S E Q S Q Q P**↓**F Q P Q** P F
	FM-POP	Q P Q P F P↓Q **Q S E Q S Q Q P**↓**F Q P**↓**Q** P↓F
DQ8-Glt	AN-PEP	Q **Q G Y Y P**↓**T S P**↓**Q** Q S
	FM-POP	Q **Q G Y Y P**↓**T S P**↓**Q** Q S
DQ8-Glia	AN-PEP	S G **Q G S F Q P**↓**S Q Q** N
	FM-POP	S G **Q G S F Q P**↓**S Q Q** N

↓ Major cleavage sites
† Less efficiently cleaved peptide bonds

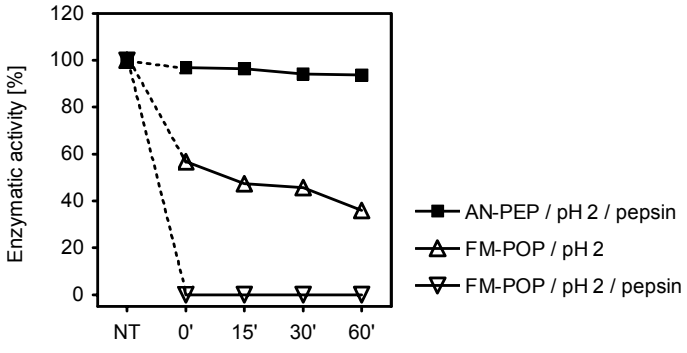


Figure 2. Resistance to low pH and pepsin digestion. AN-PEP and FM-POP were incubated at pH 2.0 with or without pepsin. At the given time points the reaction was stopped with pepstatin and the activity of both enzymes was measured at the pH optima. NT – not treated.

as this is the site where the inflammatory T cell response to gluten takes place. We therefore determined the rate of gluten peptide degradation. For this purpose we used gluten peptides corresponding to sequences found in gluten proteins from the four major gluten protein families, the α- and γ-gliadins and the high and low molecular weight glutenins. These were treated with AN-PEP or FM-POP and the reaction was stopped at various time points. Subsequently, the concentration of undegraded peptide was determined with the use of mass spectrometry. The $t_{1/2}$ values were calculated from the obtained curves (Table 3). In this set-up the $t_{1/2}$ values for AN-PEP reactions ranged between 2.4 and 6.2 minutes. In case of FM-POP these ranged from140 to 550 minutes. Thus, degradation of gluten peptides by AN-PEP was on average 60 times faster than degradation by FM-POP.

AN-PEP eliminates T cell stimulatory properties of a pepsin/trypsin digest of gluten

To determine if degradation by AN-PEP destroys the T cell stimulatory properties of peptic/tryptic digest of gluten we applied two bioassays. In the first assay we used monoclonal antibodies that are specific for T cell stimulatory sequences of α- and γ-gliadins and high and low molecular weight glutenins [14,15]. A pepsin/trypsin digest of gluten was mixed with AN-PEP at a mass ratio of 200:1 and at various time points samples were taken and tested. With the antibody-based assay, α- and γ-gliadin epitopes could no longer be detected after 30 minutes (Fig. 3A, B). Although the glutenins were cleaved at slower rate, within 120 minutes all LMW-glutenins and about 90% of HMW-glutenins were destroyed (Fig. 3C, D).

Table 2. The detected AN-PEP cleavage sites in length variants of deamidated and undeamidated Glt-156 gluten epitope. Both forms of peptides were chemically synthesized, treated with AN-PEP and the digestion products were identified by MALDI-TOF-MS. Minimal T cell stimulatory sequences given in bold.

Deamidated Glt-156 variants	Undeamidated Glt-156 variants
S Q Q Q Q P **P** ↓ **F S E E Q E S P**	S Q Q Q Q P **P** ↓ **F S Q Q Q Q S P**
Q Q Q Q P **P** ↓ **F S E E Q E S P** † **F**	Q Q Q Q P **P** ↓ **F S Q Q Q Q S P F**
Q Q Q P **P** ↓ **F S E E Q E S P** † **F** S	Q Q Q P **P** ↓ **F S Q Q Q Q S P** † **F** S
Q Q P **P** ↓ **F S E E Q E S P** † **F** S Q	Q Q P **P** ↓ **F S Q Q Q Q S P** † **F** S Q
Q P **P** ↓ **F S E E Q E S P** † **F** S Q Q	Q P **P** ↓ **F S Q Q Q Q S P** † **F** S Q Q
P **P** ↑ **F S E E Q E S P** ↓ **F** S Q Q Q	P **P** ↑ **F S Q Q Q Q S P** ↓ **F** S Q Q Q
P F S E E Q E S P ↓ **F** S Q Q Q Q	**P F S Q Q Q Q S P** ↓ **F** S Q Q Q Q

↓ Major cleavage sites
† Less efficiently cleaved peptide bonds

In the second assay we used gluten specific T cell clones specific for α- and γ-gliadin and LMW-glutenin. To evoke optimal T cell responses, most gluten peptides require modification by tissue transglutaminase. Hence, the gluten digest was first treated with tissue transglutaminase before degradation with AN-PEP at a mass ratio of 200:1 for 2 hours, after which the samples were tested with gluten-specific T cell clones. In five out of six cases the digestion of gluten with AN-PEP nullified the cellular responses (Fig. 4). Only in the case of an α-gliadin specific T cell clone approximately 5 % of the response to undigested gluten was still present in the AN-PEP treated gluten.

AN-PEP degrades intact gluten molecules

To test whether AN-PEP is capable of digesting intact gluten, we treated a gluten solution (1mg/ml) with AN-PEP (5 μg/ml) for 2 hours at 37°C, pH 4.5. Subsequently, a control gluten preparation, the AN-PEP enzyme and the AN-PEP treated gluten were separated by reverse phase HPLC (Fig. 5). The UV-traces clearly indicate the position of the AN-PEP enzyme as well as that of the digested and undigested gluten (Fig. 5A). Mass spectrometric analysis of the digested gluten fractions revealed that AN-PEP treatment generated a large number of peptides: out of 152 identified peptides 128 had a molecular mass lower than 1 kDa. The amino acid sequences of 53 of these peptides were determined showing that none of these contained the 9 amino acid core of the known T cell stimulatory gluten peptides (Data not shown). In addition we determined the efficiency of gluten degradation by SDS-PAGE analysis of the HPLC fractions that eluted at a retention time of 25 to 42

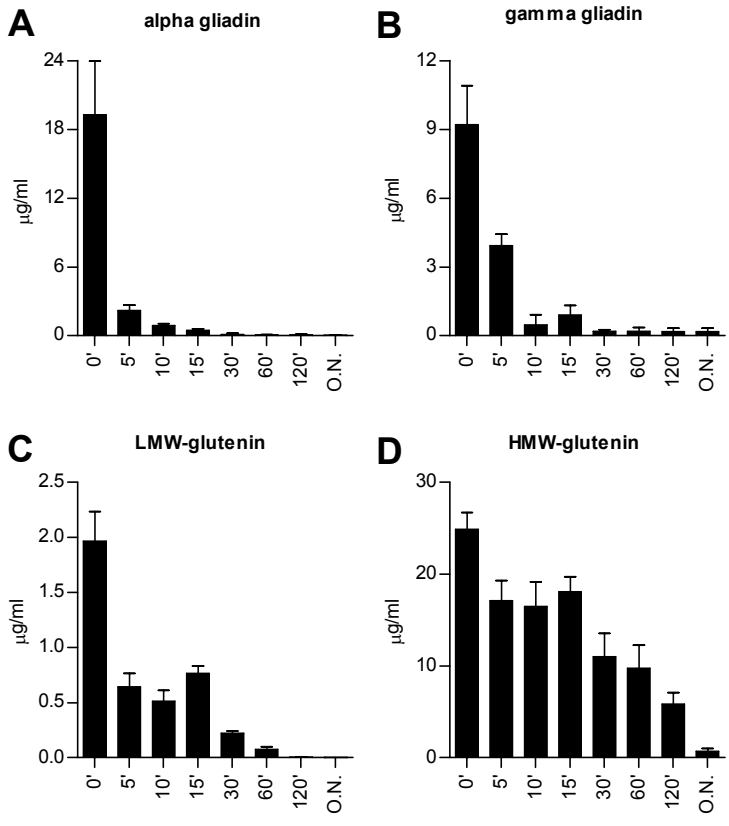


Figure 3. Degradation of peptic/tryptic gluten digest with AN-PEP. The gluten peptic/tryptic digest (0.7 mg/ml) was treated with AN-PEP (3.5µg/ml) and the load of gluten T cell epitopes was determined in a competition assay with antibodies specific for gliadins and glutenin derivedpeptides. The graphs represent the average of 2 (A, B) or 3 (C, D) separate measurements. O.N. - overnight.

minutes. Proteins were either visualized by Coomassie Brillant Blue staining (Fig. 5B) or blotted onto nitrocellulose and stained with monoclonal antibodies specific for T cell epitopes of α- and γ-gliadin and LMW-glutenin. AN-PEP treatment of gluten resulted in the degradation of proteins with a molecular weight corresponding to intact gliadins and LMW-glutenins (33-37 kDa). The Western blot analysis also indicates the disappearance of proteins that are specifically detected with monoclonal antibodies specific for α-gliadin (Fig. 5C), γ-gliadin and LMW-glutenin

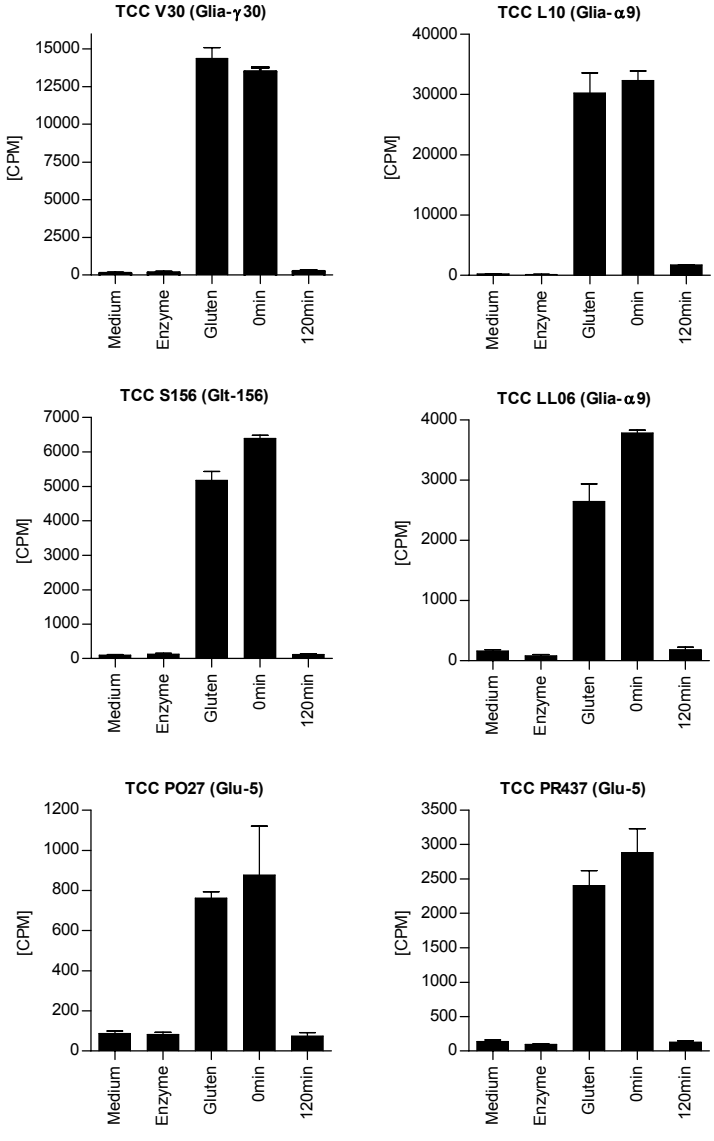


Figure 4. T cell responses to the peptic/tryptic digest of gluten treated with AN-PEP. A peptic/ /tryptic digest of gluten was treated with AN-PEP for 120 minutes (enzyme-substrate mass ratio 1:200) and tested with 6 gluten specific T cell clones. The proliferative responses were measured by ^{3}H-thymidine incorporation.

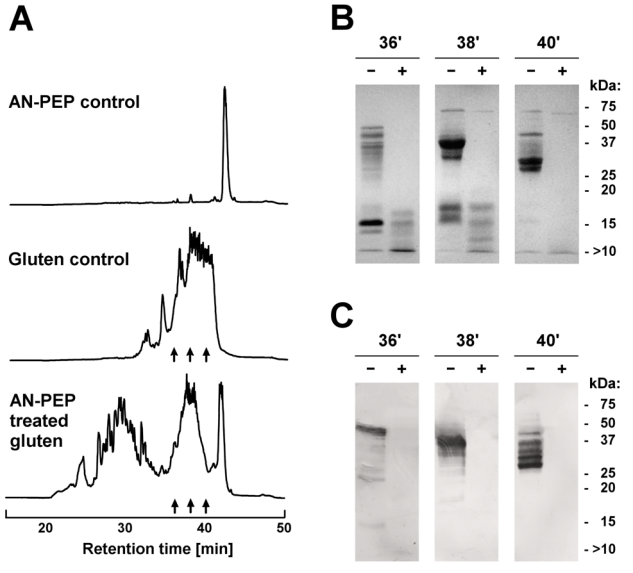
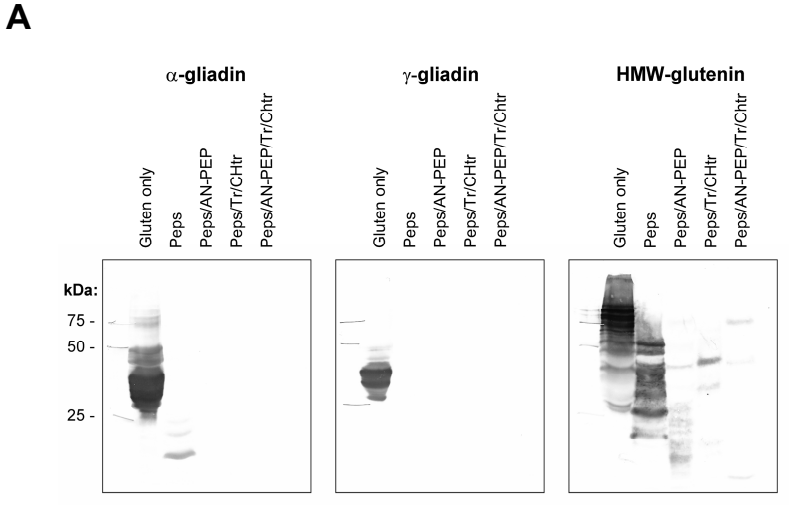


Figure 5. Degradation of intact gluten. Intact gluten (1 mg/ml) was digested with AN-PEP (5µg/ml) for 2 hours at 37°C and separated by reverse phase HPLC. The elution profiles at 214 nm of the AN-PEP control, gluten control, whole gluten digested with AN-PEP are shown (A). Fractions that eluted at a retention time of 36, 38 and 40 minutes (marked with arrows) were separated on a 15% polyacrylamide gel and either stained with Coomassie Brillant Blue (**B**) or transferred onto nitrocellulose and stained with a monoclonal antibody specific for γ-gliadins (**C**). HPLC fractions of untreated gluten are marked with "-" and fractions of gluten digested with AN-PEP marked with "+".



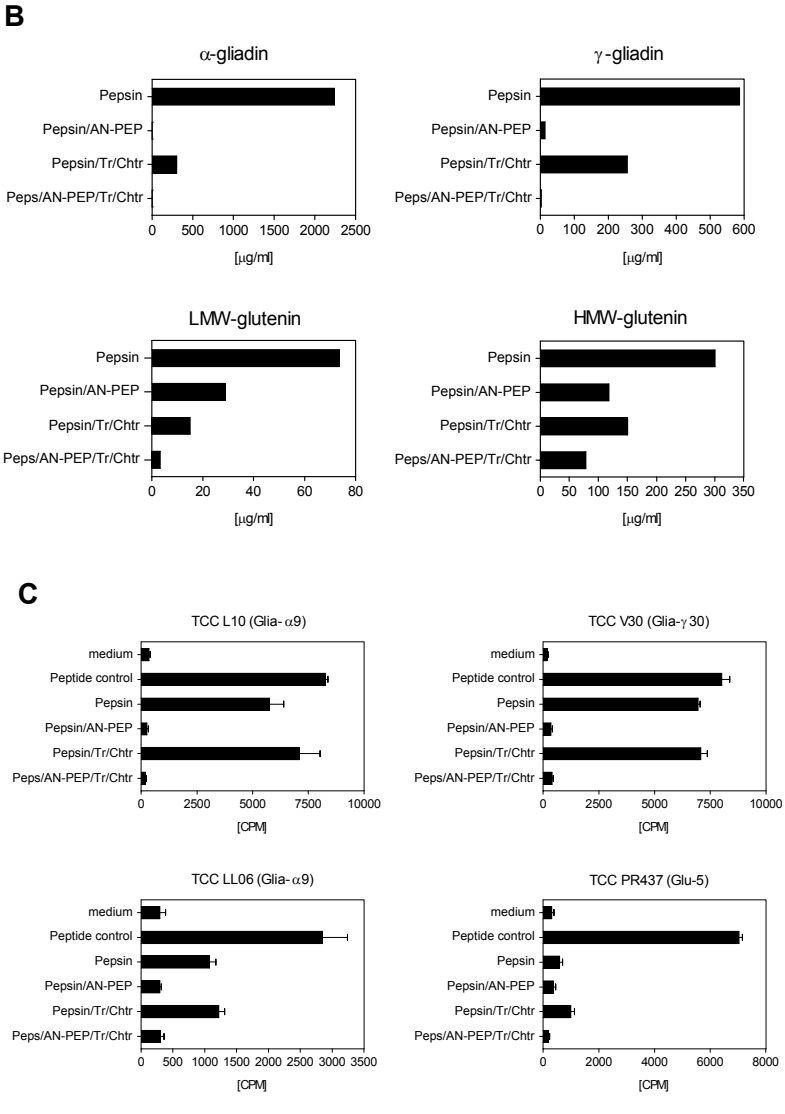


Figure 6. Digestion of whole gluten in the experimental setup mimicking the conditions present in the human GI tract. 100 ml of 2 % gluten suspension was digested with 20 mg of AN-PEP and 30 mg of pepsin for 1 hour at 37°C, pH 4.5. Subsequently the pH was adjusted to 2.0 with HCl,additional 30 mg of pepsin was added and the suspension was further incubated. After 1 hour the pH was adjusted to 7.9 with NaOH; trypsin (20 mg) and chymotrypsin (20 mg) were added. This was incubated for 1 hour at 37°C and boiled for 10 minutes to inactivate the enzymes. Similarly, the controls with only pepsin, pepsin/AN-PEP and pepsin/trypsin/chymotrypsin were prepared. The samples were visualized on Western blots with antibodies specific for α- and γ-gliadins as well as HMW -glutenins **(A).** In addition the samples were tested for the presence of gluten T cell stimulatory epitopes with either competition assay **(B)** or, after deamidation with tTG, with T cell proliferation test **(C)**.

(not shown) as the result of the AN-PEP treatment. Thus, AN-PEP can effectively breakdown intact gluten molecules into non-immunogenic peptides.

In order to beter mimic the conditions present in the human gastrointestinal tract we prepared a gluten suspension and digested it simultaneuously with pepsin and AN-PEP for one hour at pH 4.5. Simulating the acidification of gastric juice during digestion we lowered pH to 2.0 and supplied additional pepsin, which under physiological conditions is contineously being secreted. After an hour incubation gastric emptying was simulated by adjusting the pH to 7.9 and addition of trypsin and chymotrypsin. Following an hour incubation we boiled the samples to inactivate the enzymes and tested the completeness of gluten degradation with gluten-specific antibodies and patient-derived gluten-specific T cells. The SDS-PAGE separation and Western blotting analysis of gluten digest suspensions revealed very efficient degradation of α- and γ-gliadin molecules by pepsin, which was additionaly aggravated by AN-PEP (Fig. 6A). After the incubation with both enzymes we were not able to detect any gluten epitopes in fragments of gliadin with molecular mass above 10 kDa. HMW-glutenins were less efficiently cleaved by pepsin; still AN-PEP remarkably enhanced the degradation. Additional treatment with trypsin and chymotrypsin left intact only trace amounts of the starting material. To further investigate the efficiency of gluten degradation with AN-PEP we tested the digests in the competition assays with antibodies directed against α- and γ-gliadins as well as LMW and HMW-glutenins (Fig. 6B). As expected, AN-PEP very efficiently cleaved gliadin epitopes whereas glutenins proved more resistant to the proteolysis and were degraded at slower rate.

In the second assay we used gluten specific T cell clones specific for α- and γ- gliadin, and Glu 5 - a gluten epitope of unknown origin. To evoke optimal T cell responses, most gluten peptides require modification by tissue transglutaminase, therefore, the gluten digests were first treated with tissue transglutaminase, after which the samples were tested with gluten-specific T cell clones. In all the cases the digestion of gluten with pepsin and AN-PEP virtually nullified the cellular responses (Fig. 6C).

DISCUSSION

Presently the only treatment for celiac patients is a life-long gluten-free diet. Strict adherence to this diet is expensive and arduous. Also, contamination of the naturally gluten free products with traces of gluten can be detrimental to patients. It has been shown that only 100 milligram of gluten or similar proteins from rye and barley a day can result in villous atrophy [16]. Oral supplementation with post-proline cutting enzymes could be a valuable method to eradicate the proline-rich

T cell stimulatory epitopes from gluten-proteins. Ideally, the degradation of gluten should occur in the stomach, before gluten or gluten fragments can reach the upper duodenum where gluten specific T cell reside in the lamina propria. The prolyl oligopeptidases suggested in literature, however, have limitations in this respect as they are (i) not stable at the low pH of gastric juice [8] (ii) susceptible to digestion with pepsin [8] (iii) characterized by a preference for small substrates [10] (iv) and not efficient enough to cope with the amount of gluten present in a normal diet [11]. Also encapsulation of the traditional prolyl oligopeptidases to protect them against gastric juice, as proposed by Gass et al. [9], will be ineffective as the gluten will not be degraded before it reaches the proximal part of the duodenum, the site where gluten induces inflammatory T cell responses.

We studied a recently identified prolyl endoprotease from *A. niger*, AN-PEP, and demonstrate that this enzyme does not suffer from these limitations and is able to degrade gluten under conditions found in the stomach. After consumption of a meal the pH of the stomach lumen is transiently neutralized. Subsequently, accelerated production of gastric juice causes a slow reacidification. While the pH is decreasing due to the hydrochloric acid secretion, the proteolytic activity of pepsin increases. We observed that AN-PEP is active at the whole pH range present in the stomach (with the pH optimum between 4.0 and 5.0). At the same time AN-PEP is fully resistant to low pH and degradation by pepsin present in the gastric juice. Furthermore, when delivered to the duodenum, the acidic and partially digested chyme is mixed with pancreatic juices which raises the pH, transiently restoring optimal conditions for the AN-PEP activity, which would further facilitate the breakdown of gluten by AN-PEP. Moreover, the introduction of cleavages into the proline-rich sequences is likely to expose new cleavage sites for pancreatic and brush-border enzymes, which would further enhance the degradation [17,18].

The efficiency of gluten degradation was measured in several experimental set-ups. First, the proteolytic breakdown of the single peptides was monitored with mass spectrometry. Secondly, we tested weather AN-PEP is capable of degrading a peptic/tryptic digest of gluten. The degradation of gluten peptides was determined in competition assays with antibodies specific for T cell epitopes of α- and γ-gliadins as well as HMW- and LMW-glutenins and in T cell proliferation tests. The results of these experiments demonstrated that AN-PEP is highly efficient in degradation of both gliadin and glutenin epitope sequences in complex mixtures. The AN-PEP treatment led to complete degradation of the T cell epitopes in almost all cases. In the third approach we tested if AN-PEP can degrade intact gluten molecules. For this purpose solubilized whole gluten, treated it with AN-PEP and evaluated the digestion by mass spectrometry as well as by SDS-PAGE followed by Western blotting with antibodies against gluten T cell epitopes. The results demonstrate that AN-PEP is highly efficient in degradation of both gliadin and glutenin molecules

and that the AN-PEP treatment led to complete degradation of the T cell epitopes in almost all cases. This is in contrast to prolyl oligopeptidases, which are inefficient in cleaving large peptides and intact proteins. Also, contrary to a previous studies on gluten detoxification, in which sequential digestion with a number of gastric, pancreatic and brush border proteases preceded or followed the treatment with prolyl oligopeptidase [18], our data shows that digestion with AN-PEP alone is sufficient to eliminate the majority of the toxic sequences from gluten.

To better mimic the physiological conditions present in the stomach we have also treated a gluten suspension with AN-PEP in the presence of pepsin at pH 4.5 followed by acidification to pH 2.0. Subsequently we raised the pH to 7.9 and added trypsin and chymotrypsin to simulate gastric emptying. The breakdown of gluten was monitored with SDS-PAGE and Western blotting, competition assay with antibodies specific for α- and γ-gliadins as well as LMW and HMW-glutenins and patient-derived gluten-specific T cell clones. The results indicated the highly efficient degradation of α- and γ-gliadins. The cleavage of glutenins was at slower rate comparing to gliadins. This could be due to the fact that on average the glutenins contain less proline residues compared to the gliadins. Moreover, the sequences recognized by the gluten specific antibodies antibodies are shorter (5-6 amino acid residues) than T cells epitopes (9-10 amino acids). Thus, measurements with these antibodies can lead to an overestimation of the amount of toxic sequences left. The occurrence of this phenomenon is supported by the observation that gluten treated with AN-PEP was not able to stimulate proliferation of a T cell clone specific for LMW-glutenin. Finally, the majority of gluten-specific T cell responses in celiac patients are directed against gliadin epitopes [19,20]. Thus, it is conceivable that celiac patients could tolerate higher concentrations of glutenins than gliadins. Finally, we observed that AN-PEP on average is 60 times more efficient in cleaving gluten peptides compared to FM-POP, an observation that appears highly relevant as the majority of T cell stimulatory gluten peptides need to be broken down before they reach the small intestine.

In conclusion, we demonstrate that the prolyl endopeptidase from *Aspergillus niger* can act under conditions similar to those found in the gastrointestinal tract, and is capable of degrading intact gluten molecules and T cell stimulatory epitopes from gluten into harmless fragments. The enzyme is extremely stable and can be produced at low cost at food grade quality in an industrial setting [12]. Since no animal model for celiac disease is currently available the in vivo efficacy of AN-PEP for gluten detoxification will ultimately have to be addressed in clinical studies involving celiac patients. AN-PEP appears to be a prime candidate for such clinical trials.

ACKNOWLEDGEMENTS

We thank dr. Bart Roep and dr. Jeroen van Bergen for critical reading of the manuscript, dr. Jan Wouter Drijfhout and Willemien Benckhuijsen for peptide synthesis.

GRANTS

This study was supported by the Netherlands Organization for Scientific Research (grant 912-02-028), the Celiac Disease Consortium, an Innovative Cluster approved by the Netherlands Genomics Initiative and partially funded by the Dutch Government (BSIK03009), and the Centre for Medical Systems Biology, a center of excellence approved by the Netherlands Genomics Initiative/Netherlands Organisation for Scientific Research.

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