Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease

Dariusz Stepniak,1 Liesbeth Spaenij-Dekking,1 Cristina Mitea,1 Martine Moester,1 Arnoud de Ru,1 Renee Baak-Pablo,1 Peter van Veenen,1 Lупpo Edens,2 and Frits Koning1

1Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden; and 2DSM Food Specialties, Delft, The Netherlands

Submitted 20 January 2006; accepted in final form 7 May 2006

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 (9, 17, 18). Subsequently, these peptides bind to either human leukocyte antigen (HLA)-DQ2 or -DQ8 molecules and evoke T cell responses leading to inflammation in the small intestine, ultimately leading 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 (20). Because human gastric and pancreatic enzymes lack postproline 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 nonhuman proteases for gluten detoxification was already proposed in the late 1950s (5), and a clinical trial took place in 1976 (8) 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 (14). Prolyl oligopeptidases from Sphingomonas capsulate and Myxococcus xanthus were also studied and have comparable properties (4, 13). Prolyl oligopeptidases, however, have optimum pH between 7 and 8, so they cannot function at the acid pH in the stomach. Also, they are efficiently broken down by pepsin (13). Besides, due to their structure in which a β-propeller domain restricts entry into the active center, the enzymes preferentially cleave short peptides (12). 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. (7).

In the present study, we have investigated a newly discovered prolyl endoprotease from Aspergillus niger (AN-PEP) (3). 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 oligopeptidases. 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. Because 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 to be a good candidate for such studies.

MATERIALS AND METHODS

Reagents. Pepsin (2,331 U/mg), trypsin (9,600 U/mg), chymotrypsin (54 U/mg), guinea pig tissue transglutaminase (1.68 U/mg), pepstatin A, PMSF, and standard 4-nitroaniline (pNA) were from Sigma (St. Louis, MO). FM-POP (35 U/mg) was from ICN Biochemi-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
cals (Aurora, OH). AN-PEP was produced and purified by DSM Food Specialties (Delft, The Netherlands). Besides postprolline 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-coumarinylamide (AMC) were from Fluka Chemie (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 (2–6 pH), Tris·HCl (6–8 pH), and glycine/NaOH (8–12 pH). 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 min at 37°C. The released AMC was measured fluorometrically at λex 360 nm and λem 460 nm using a CytFluor multi-well plate reader (PerSeptive Biosystems, Framingham, MA).

**Stability at low pH and resistance to pepstatin degradation.** Both AN-PEP and FM-POP were diluted with 100 mM glycine/HCl buffer (2.0 pH) to 1 mg/ml and mixed with an equal volume of 100 mM sodium acetate buffer (pH 7.0). The concentration of both AN-PEP and FM-POP was 32 μg/ml and 0.2 μg/ml, respectively. The reaction was carried out for 30 min at 37°C. The released AMC was measured fluorometrically at λex 360 nm and λem 460 nm using a CytFluor multi-well plate reader (PerSeptive Biosystems, Framingham, MA).

**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, NY). Every measurement was performed in duplicate. The enzyme samples were diluted in 100 mM Tris·HCl buffer (7.0 pH) to a final concentration of 0.1 μM. The reaction was started by mixing 95 μM enzyme with 5 μl of substrate (4 μM in 60% methanol). After 30 min at 37°C, the reaction was stopped with 50 μl of 1 M 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 (4.5 pH) to a final concentration of 0.1 μM. The reaction was started by mixing 50 μM enzyme with 50 μM of substrate (40 μM in 100 mM sodium acetate buffer, pH 4.5). After 30 min at 37°C the absorption at 405 nm was measured using an ELISA plate reader (Spectro Classic, Wallac).
sin (20 mg) were added. This was incubated for 1 h at 37°C and boiled for 10 min 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 6× protein sample buffer [60% glycerol, 300 mM Tris (pH 6.8), 12 mM EDTA (pH 8.0), 12% SDS, 864 mM 2-mercaptoethanol, and 0.05% bromophenol blue] and run on a 12.5% SDS-PAGE gel. The proteins were visualized either directly using Imperial protein stain (Pierce) or after transfer to nitrocellulose membranes with the MAbs specific for stimulatory T cell epitopes from α- and γ-gliadins and HMW- and LMW-glutenins (15, 16).

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 (15, 16). Briefly, microtiter plates (Nunc, Copenhagen, Denmark) were incubated overnight with 2–5 μg/ml MAbs 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). Of the containing-samples, different dilutions were made in 20 mM NaH2PO4/Na2HPO4 (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 from 10 μg/ml to 10 ng/ml. For the LMW-glutenin assay, a standard curve was made using the synthetic peptide QPPFSQQQQP-PFSQQQQPSFSQQQ-amine in a concentration range from 1 μg/ml to 1 ng/ml. For the HMW-glutenin assay, a standard curve was made using a trypsin/chymotrypsin digest of recombinant HMW-glutenin proteins (provided by P. Shewry; Rothamsted Research, Hampenden, UK) in a concentration range from 1 μg/ml to 1 mg/ml. The assays were repeated at least twice.

T-cell proliferation assay. The gluten digest samples were thawed, centrifuged for 10 min at 18,000 g, and incubated with guinea pig tissue transglutaminase (200 μg/ml) and CaCl2 (10 mM) for 1 h 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 105 gluten-specific T cells stimulated with 105 irradiated HLA-DQ2-matched allogeneic peripheral blood mononuclear cells (3,000 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 h at 37°C, cultures were pulsed with 0.5 μCi of [3H]thymidine and harvested 18 h later; thymidine incorporation was quantified with a liquid scintillation counter.

RESULTS

AN-PEP is active at pH present in the stomach. To determine the pH optimum of AN-PEP, the enzyme was incubated with the 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 4 and 5. In contrast, the pH optimum of FM-POP is about pH 7–8, and the enzyme is not active at a pH below 5.

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 min, the pepsin was inactivated by the addition of the inhibitor pepstatin A, and the remaining enzyme activity in the samples was determined at the optimum pH 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 min at pH 2.0 reduced its activity by ~50%, whereas 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 and Supplementary Data Set 1 (available at the American Journal of Physiology-Gastrointestinal and Liver Physiology web site)]. 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 proteolyzed (Table 3). In general, peptide bonds located in the
middle of a peptide were more efficiently cleaved than those located near the NH₂ or COOH 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 h. It is crucial that most of the toxic gluten sequences are destroyed before reaching the duodenum 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 min. In the case of FM-POP, these ranged from 140 to 550 min. 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 whether degradation by AN-PEP destroys the T cell stimulatory properties of peptic/trypsin digest of gluten, we applied two bioassays. In the first assay we used MAbs that are specific for T cell stimulatory sequences of gluten– and gliadin epitopes could no longer be detected after 30 min (Fig. 3, A and B). Although the glutenins were cleaved at a slower rate, within 120 min, all LMW-

Table 2. The detected AN-PEP cleavage sites in length variants of deamidated and undeamidated Glit-156 gluten epitope

<table>
<thead>
<tr>
<th>Deamidated Glit-156 Variants</th>
<th>Undeamidated Glit-156 Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQQQQPQ</td>
<td>FSQQQESP</td>
</tr>
<tr>
<td>QQPPP</td>
<td>FSSEQESP</td>
</tr>
<tr>
<td>QQPPP</td>
<td>FSSEQESP</td>
</tr>
<tr>
<td>QPPP</td>
<td>FSSEQESP</td>
</tr>
<tr>
<td>PPP</td>
<td>FSQQQESP</td>
</tr>
<tr>
<td>PFSEEQESP</td>
<td>FSQQQESP</td>
</tr>
</tbody>
</table>

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 are given in bold. Arrows indicate cleavage sites. †Less efficiently cleaved peptide bonds.
The immunoogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The amino acid sequences of 53 of these peptides were determined, showing that none of these contained the nine-amino-acid core of the known T cell stimulatory gluten peptides. The amino acid sequences of 53 of these peptides were determined, showing that none of these contained the nine-amino-acid core of the known T cell stimulatory gluten peptides. The amino acid sequences of 53 of these peptides were determined, showing that none of these contained the nine-amino-acid core of the known T cell stimulatory gluten peptides.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.

The immunogenic gluten peptides (2 mg/ml) were treated with either AN-PEP or FM-POP (1 μg/ml) at their optimum pH. The concentration of the undigested peptide at the time points of 0, 5, 10, 30, 60, and 120 min was determined by Q-TOF mass spectrometry. The t_{1/2} values were calculated from the obtained curves.
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 mg of gluten or similar proteins from rye and barley per day can result in villous atrophy (2). Oral supplementation with postproline 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 1) not stable at the low pH of gastric juice (13), 2) susceptible to digestion with pepsin (13), 3) characterized by a preference for small substrates (12), and 4) not efficient enough to cope with the amount of gluten present in a normal diet (7). Also, encapsulation of the traditional prolyl oligopeptidases to protect them against gastric juice, as proposed by Gass et al. (4), 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 endopeptidase 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. Although 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 entire pH range present in the stomach (with the pH optimum between 4 and 5). 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 (6, 11).

The efficiency of gluten degradation was measured in several experimental setups. First, the proteolytic breakdown of the single peptides was monitored with mass spectrometry. Secondly, we tested whether 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 H9251- and H9253-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 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 previous studies (6) 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, our data show 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 H9251- and H9253-gliadins, 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 a slower rate compared with gliadins. This could be due to the fact that, on average, the glutenins contain less proline residues compared with the gliadins. Moreover, the sequences recognized by the gluten-specific 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 (1, 10). 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 with 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 \textit{A. 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 (3). Because 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.

ACKNOWLEDGMENTS

We thank B. Roep and J. van Bergen for a critical reading of the manuscript and J. W. Drijfhout and W. Benckhuijsen for the 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.

REFERENCES


