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

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Am J Physiol Gastrointest Liver Physiol 291: G621–G629, 2006. First published May 11, 2006; doi:10.1152/ajpgi.00034.2006.—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 endopeptidase, and the destruction of the T cell epitopes was tested using mass spectrometry, T cell proliferation assays, ELISA, reverse-phase HPLC, SDS-PAGE, and Western blotting. We observed that the A. niger prolyl endopeptidase works optimally at 4–5 pH, remains stable at 2 pH, 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 endopeptidase 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 endopeptidase can be used as an oral supplement to reduce gluten intake in patients.

Immune response; T cell tolerance 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 endopeptidase, and the destruction of the T cell epitopes was tested using mass spectrometry, T cell proliferation assays, ELISA, reverse-phase HPLC, SDS-PAGE, and Western blotting. We observed that the A. niger prolyl endopeptidase works optimally at 4–5 pH, remains stable at 2 pH, 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 endopeptidase 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 endopeptidase can be used as an oral supplement to reduce gluten intake in patients.

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; diarrhea, 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 endopeptidase 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-

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mixture and added to either 40 μl of 1 M NaCl (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 vol/vol vol 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/trypsin gluten digest.** The gliadin peptic/trypsin digest was prepared as described (19). The obtained preparation was deamidated with guinea pig tissue transglutaminase (100 μg/ml; Sigma) in PBS with 2 mM CaCl2 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 min at 37°C in a total volume of 520 μl. The reaction was stopped by the addition of 5 μl of 1 M NaOH after which the pH was adjusted to 7. Control samples were prepared by mixing the peptic/trypsin 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/trypsin digest of gluten was treated with AN-PEP in the following way: 2 ml of gluten peptic/trypsin digest (4 mg/ml) were mixed with 2 ml of 100 mM citrate buffer (pH 4.5) and incubated for 5 min at 37°C. Subsequently, 40 μl of AN-PEP (1 mg/ml) was added, and at time points 5, 10, 15, 30, 60, and 120 min and 20 h, the digestion was stopped by removing 400-μl aliquots of the samples to vials containing 10 μl of 10 M NaOH after which the pH was adjusted to 7. Control samples were prepared by mixing the peptic/trypsin 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 gliadins was determined.

**Digestion of whole gluten: protocol 1.** One gram of gluten (Sigma) was suspended in 20 ml of 1 M acetic acid and ultrasonicated for 30 min. Insoluble material was removed by centrifugation for 15 min at 3,000 rpm. Subsequently, the pH of the supernatant was adjusted to 4.5 with NaOH, and the resulting precipitate was removed by centrifugation for 15 min at 3,000 rpm. Protein concentration in the supernatant was determined with a Bradford assay and adjusted to 1 mg/ml by the addition of 100 mM citrate buffer (pH 4.5). Subsequently, 5 μl of AN-PEP (1 mg/ml) was added to 1 ml of the gluten solution and incubated for 2 h 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 reverse-phase HPLC 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). Individual peptides were sequenced with an ion trap mass spectrometer (HCTplus; Bruker Daltonics, Bremen, Germany). Several fractions collected at a retention time of 25–42 min were applied to 15% SDS-PAGE under reducing conditions. The proteins were either visualized with Coomassie blue (Imperial protein stain; Pierce, Rockford, IL) or transferred to nitrocellulose for subsequent Western blotting with MAb specific for α- and γ-gliadin and high molecular weight (HMW)- and low molecular weight (LMW)-gliadins as described (15, 16).

**Digestion of whole gluten: protocol 2.** Two grams of gluten (Sigma) 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 a 1-h incubation at 37°C, the pH was adjusted to 2.0 with HCl; an 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 chymotryp-
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-gliadin 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 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). Of the gluten-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 of 10 μg/ml to 10 ng/ml. For the LMW-glutenin assay, a standard curve was made using the synthetic peptide QPPFSQQQQP-PFSQQQQPFSQQQQQ-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, Hampden, UK) in a concentration range from 1 μg/ml to 1 ng/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 glutenspecific 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...

![Fig. 1. Comparison of the pH optima of prolyl endoprotease from Aspergillus niger (AN-PEP) and pepsin/trypsin/chymotrypsin (FM-POP). The hydrolytic activity of the enzymes was measured fluorimetrically with the fluorogenic substrate Z-Gly-Pro-AMC.](http://ajpgi.physiology.org/)

![Fig. 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 optimum pH.](http://ajpgi.physiology.org/)
The most prominent cleavage sites of AN-PEP and FM-POP in selected T cell stimulatory gluten peptides are given in bold. Arrows indicate cleavage sites. †Less efficiently cleaved peptide bonds.

The detected AN-PEP cleavage sites in length variants of deamidated and undeamidated Glt-156 gluten epitope

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 1/2 values were calculated from the obtained curves (Table 3). In this set-up, the 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 used MAbs that are specific for T cell stimulatory sequences of α- and γ-gliadins and high and low molecular weight glutenins (15, 16). 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 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

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 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.

In the second assay we used gluten-specific T cell clones specific for \( \alpha \)- and \( \gamma \)-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 h, 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 \( \alpha \)-gliadin-specific T cell clone, \( \sim 5\% \) 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 (1 mg/ml) with AN-PEP (5 µg/ml) for 2 h 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 \(<1\) kDa. 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 (Supplementary Data Set 2). 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 min. Proteins were either visualized by Coomassie blue staining or blotted onto nitrocellulose and stained with MAb specific for T cell epitopes of \( \alpha \)- and \( \gamma \)-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 MAbs specific for \( \gamma \)-gliadin (Fig. 5C), \( \alpha \)-gliadin, and LMW-glutenin (not shown) as the result of the AN-PEP treatment. Thus AN-PEP can effectively breakdown intact gluten molecules into nonimmunogenic peptides.

**Table 3. Kinetics of degradation of gluten peptides**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>( t_{1/2} ), min</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )-Gliadin (Glia-( \alpha ))</td>
<td>QLQPFQPQLPY</td>
<td>3.87 141.56</td>
</tr>
<tr>
<td>( \gamma )-Gliadin (Glia-( \gamma ))</td>
<td>QPQPFQPQSFQPQRF</td>
<td>2.36 207.21</td>
</tr>
<tr>
<td>LMW-Glt (Glt-156)</td>
<td>QPPFSQOQPSPQFQ</td>
<td>5.80 552.49</td>
</tr>
<tr>
<td>HMW-Glt</td>
<td>QQQYYPSPQSGQ</td>
<td>6.19 226.45</td>
</tr>
</tbody>
</table>

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.

To better mimic the conditions present in the human gastrointestinal tract, we prepared a gluten suspension and digested it simultaneously with pepsin and AN-PEP for 1 h at pH 4.5. Simulating the acidification of gastric juice during digestion, we lowered the pH to 2.0 and supplied additional pepsin, which under physiological conditions is contiously being secreted. After an hour, incubation gastric emptying was simulated by adjusting the pH to 7.9 and by 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 \( \alpha \)- and \( \gamma \)-gliadin molecules by pepsin, which was additionally 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 a molecular mass \( \sim 10 \text{kDa} \). 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 \( \alpha \)- and \( \gamma \)-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 a slower rate.

In the second assay, we used gluten-specific T cell clones specific for \( \alpha \)- and \( \gamma \)-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).

**Fig. 3. Degradation of peptic/trypsic gluten digest with AN-PEP.** The gluten peptic/trypsic digest (0.7 mg/ml) was treated with AN-PEP (3.5 µg/ml), and the load of gluten T cell epitopes was determined in a competition assay with antibodies specific for gliadins and glutenin-derived peptides. The graphs represent the average of 2 (A and B) or 3 (C and D) separate measurements. O.N., overnight; HMW, high molecular weight; LMW, low molecular weight.
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 endoprotease from A. niger, AN-PEP, and demonstrate that this enzyme does not

![Fig. 4. T cell responses to the peptic/trypic digest of gluten treated with AN-PEP. A peptic/trypic digest of gluten was treated with AN-PEP for 120 min (enzyme-substrate mass ratio 1:200) and tested with 6 gluten-specific T cell clones. The proliferative responses were measured by [3H]thymidine incorporation.](http://ajpgi.physiology.org)
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 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 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 α- and γ-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-gliadin. 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 glutens than gliadins.

Finally, we observed that AN-PEP, on average, is 60 times more effective than pepsin in the degradation of gluten. The experimental setup mimicking the conditions present in the human gastrointestinal tract is as follows. One hundred milliliters of 2% gluten suspension were digested with 20 mg of AN-PEP and 30 mg of pepsin for 1 h at 37°C, pH 4.5. Subsequently, the pH was adjusted to 2.0 with HCl, an additional 30 mg of pepsin was added, and the suspension was further incubated. After 1 h, the pH was adjusted to 7.9 with NaOH; trypsin (Tr; 20 mg) and chymotrypsin (Chtr; 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 visualized on Western blots with antibodies specific for α- and γ-gliadins as well as HMW-glutenin (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). Peps, pepsin.
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 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.

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