Effect of Prolyl Endopeptidase on Digestive-Resistant Gliadin Peptides in Vivo

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ABSTRACT

Many gluten peptides elicit proliferative responses from T cells from Celiac Sprue patients, influencing the pathogenesis of this small intestinal disorder. These peptides are Pro- and Gln-rich in character, suggesting that resistance to proteolysis promotes their toxicity. To test this hypothesis, we analyzed the digestive resistance of a panel of α- and γ-gliadin peptides believed to induce toxicity via diverse mechanisms. Most were highly resistant to gastric and pancreatic protease digestion, but they were digested by intestinal brush-border peptidases. In some instances, there was accumulation of relatively long intermediates. Control peptides from gliadin and myoglobin revealed that digestive resistance depended on factors other than size. Prolyl endopeptidase (PEP) supplementation substantially reduced the concentrations of these peptides. To estimate a pharmacologically useful PEP dose, recombinant PEP was coperfused into rat intestine with the highly digestive-resistant 33-mer peptide LQLQPF(PQPQLPY)3PQPQPF (PEP:peptide weight ratio 1:50 to 1:5). PEP dosing experiments indicate significant changes in the average residence time. The in vivo benefit of PEP was verified by coperfusion with a mixture of 33-mer and partially proteolyzed gliadin. These data verify and extend our earlier proposal that gliadin peptides, although resistant to proteolysis, can be processed efficiently by PEP supplementation. Indeed, PEP may be able to treat Celiac Sprue by reducing or eliminating such peptides from the intestine.
al., 2002). This led to the proposal that proteolytic resistance of gliadin peptides contributes to their toxicity by allowing their intestinal luminal accumulation. In this study, we provide the first quantitative data that support this hypothesis. By comparing the in vitro and in vivo digestion of a panel of structurally diverse α- and γ-gliadin-derived peptides that are believed to induce toxic effects in Celiac Sprue, we were able to quantify their relative resistance to digestion. The experimentally measured parameters were used to develop a quantitative model for the luminal content of gliadin peptides in the human intestine. Our findings indicate that these peptides are able to quantify their relative resistance to digestion. The proteolyzed at rates comparable with nonimmunogenic control peptides.

Materials and Methods

Materials. A summary of the peptides used in the following experiments, the source, and peptide identifiers can be found in Table 1. Throughout this report, peptides are identified by their sequence or numerical peptide identifiers. Purity of all peptides was verified by reverse phase HPLC. LQLQPFPQPQLPYPQQLPYPQPQLPYPQPQPF was found to contain the related 32-mer LPYPQQLPYPQLPYPQPQPF, as well as smaller quantities of pyro-QLQPFPQPQLPYPQQLPYPQPQPF, and prolyl endopeptidase was also added at a final concentration of 100 μU/μl. The reaction mixture was monitored to be at 37°C by rectal thermometer.

In Vitro Digestion of Gliadin Peptides. The in vitro digestion of each peptide was performed as follows: 75 μM (final concentration) of peptide was combined with 32 μl of 0.03 M HCl, pepsin (1:100 w/w peptide), and water to give a volume of 104 μl. The reaction was carried out for 30 min at 37°C and was stopped by the addition of 40 μl of 1 M sodium phosphate/sodium bisphosphate, pH 7.04. Chymotrypsin (1:100 (w/w)), trypsin (1:100 (w/w)), and carboxypeptidase A (1:100 (w/w)) were then added to give a final reaction volume of 168 μl. The reaction mixture was incubated at 37°C for the indicated time and then stopped by heating at 90°C for 5 min. Alternatively, the peptide (75 μM final concentration) was combined with rat intestinal brush-border enzymes, where the final concentration of brush-border aminopeptidase N was 100 μU/μl. The rat intestinal brush border was prepared as described previously (Ahnen et al., 1982), and the activity was measured by assaying continuously at 30°C in 0.1 M Tris·HCl, pH 8.0, containing 1 mM Leu-pNA [extinction coefficient at 410 nm (ε410) = 8800 M⁻¹/cm⁻¹] in 1% dimethyl sulfoxide to improve solubility. In some reactions, prolyl endopeptidase was also added at a final concentration of 100 μU/μl. The reaction mixture was analyzed by reverse phase HPLC on a Vydac 218MS54 column (4.6 mm inner diameter × 15 cm).

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The rate of substrate disappearance for the peptides was calculated as detailed previously (Smithson and Gray, 1977).

It is important to note that the in vivo experiments presented here are designed to represent the physiological assimilation of gliadin peptides that are end products of pancreatic protease digestion. Consequently, our perfusion model has been simplified to exclude the effects of endogenous pancreatic enzymes and pancreatic protein secretions. We recognize that pancreatic enzymes are likely to degrade orally administered PEP, and additional intestinal luminal proteins are likely to compete with the PEP's ability to proteolyze P5. However, our intention at this stage of the work was not to establish the stability of PEP to pancreatic digestion but instead to understand quantitatively the resistance of potentially toxic gliadin peptides to brush-border proteolysis, as well as the effect of PEP supplementation on this process under ideal intraintestinal conditions.

Mathematical Modeling of Intestinal Content of Gliadin Peptides. The in vivo data presented in this report was used to estimate the luminal content of plugs of gliadin peptides P4 and P5 as they progress through the small intestinal tract. The beneficial effect of PEP supplementation on P5 processing was also analyzed in a similar manner. In this analysis, the length and diameter of the human small intestine were assumed to be 5 m and 3.8 cm, respectively, and the transit time of a peptide plug across this distance was estimated as 4 h. For analytical purposes, the intestine was divided into 240 segments of ~2.1-cm length each, such that each segment corresponded to the distance traversed per minute. To estimate the peptide concentration, C, that exits each segment i, it is first necessary to determine the rate constant, k, for intestinal brush-border membrane-catalyzed proteolysis of each peptide. The rate constant k was assumed to be first order, which would be the case if the Michaelis-Menten parameter for brush-border membrane (BBM) peptidases have $K_M \gg [S]$. This assumption was validated for GLGG (a peptide easily digested by intestinal brush-border peptidases) in an earlier study (Smithson and Gray, 1977).

The rate constant k was calculated in an iterative manner for each peptide. The correct k value was that at which the calculated surface rate of peptide proteolysis (i.e., moles peptide per unit surface area per unit time) was four times that observed experimentally in the rat intestinal perfusion loop. (The surface area in the human small intestine is 300-fold greater than that of a smooth cylindrical tube, whereas the surface area of the rat small intestine is 75-fold higher than that of a smooth tube (Fisher and Parsons, 1950; Ganong, 1979; Ferraris et al., 1989).) It should be noted that these surface rates of peptide proteolysis are substantially greater (>8 times) than the rate of movement across the epithelium (Matsysik-Budnik et al., 2003). Consequently, the flux of peptide across the epithelium could be safely ignored in this study without affecting its conclusions.

Results

Digestive Resistance of Gliadin Peptides and Effect of PEP. The gliadin peptides P2-P8 (Table 1) were incubated with pepsin, trypsin, chymotrypsin, carboxypeptidase A, and elastase and purified rat intestinal BBMs as detailed previously (Shan et al., 2002) to assess their resistance to digestion by rat intestinal surface peptidases, we performed a series of in vivo peptide digestion experiments. Peptides were perfused at physiologically relevant concentra-
tions via a catheter placed 5 cm distal to the ligament of Treitz, and samples were collected from a catheter positioned 20 cm distally. The peptide GLGG, known to be efficiently assimilated by the small intestine (Smithson and Gray, 1977), was used as a control to determine variations between rats and as a standard to compare to published data. GLGG was perfused at the beginning and end of each experiment to determine any loss of in vivo digestive capacity during the in vivo procedure. The rate of GLGG disappearance was determined to be 74 ± 7 pmol/cm² s at the beginning of the perfusion experiment, and this disappearance rate did decline at the end of the experiment to 49 ± 5 pmol/cm² s.

Figure 2 shows the rate of disappearance for the gliadin-derived peptides P3, P4, and P5, as determined by three different peptide perfusion experiments taking into account the decline, assumed to be linear, in intestinal capacity over the period of the experiment, as detailed above. Also included in Fig. 2 are the rate data for P8. P10 (a product of pepsin and pancreatic protease digestion of myoglobin) and P9 (a product of pepsin and pancreatic protease digestion of a-gliadin that lacks immunogenicity) were used as physiologically relevant controls to determine whether the size of the peptide plays a role in digestion. There currently are no known physiologically relevant controls available for comparison with P5 and P8 peptides. The proteolysis rates for P10 and P9 were 18 and 21 pmol/cm² s, respectively. All data were collected under conditions where <50% of each peptide disappeared, thereby allowing for calculation of steady-state rates of proteolysis. Together with the in vitro data in Table 1, these results establish that immunogenic gliadin peptides such as P4, P5, and P8 are not only resistant to digestion with gastric and pancreatic enzymes but also display a relative resistance to digestion by the brush-border enzymes compared with nontoxic dietary peptides such as P10 and P9.

To further explore the capacity of PEP to enhance gliadin digestion, we measured PEP supplemental proteolysis of the gliadin peptides in vivo by coperfusing the gliadin peptides with PEP, 25 µU/µl, under the same conditions as detailed above. The rate of digestion was increased significantly, 50–100%, for each gliadin-derived peptide, as shown in Fig. 2, but there was no detectable change in the rate of the control peptide (GLGG) digestion (data not shown).

HPLC analysis verified the increased digestion of the otherwise resistant gliadin peptides. Digestion of P3 produced the resistant intermediate PQPQLP (Hausch et al., 2002). No other digestive intermediates were identified for the other gliadin peptides.

To determine the amount of PEP required to eliminate P5 under the perfusion conditions described under Materials and Methods, the concentration of recombinant PEP coperfused with 50 µM P5 was systematically increased (Fig. 3). A dose-dependent reduction of P5 was observed in this experiment with virtually all of the starting material eliminated in the presence of 41.7 µg/ml PEP (∼190 µU/µl). This dose corresponds to ca. 1:5 weight ratio for PEP:P5. Under these conditions, three intermediates were observed to accumulate to ~10% of the starting material. Thus, supplementation of exogenous recombinant PEP in the jejunum can result in rapid digestion of highly proteolysis-resistant gliadin peptides such as P5.

To verify that PEP has adequate specificity so as to target P5 when present as a component of a mixture of gliadin peptides, the intestinal perfusion experiment was repeated by coperfusing 41.7 µg/ml recombinant PEP (∼190 µU/µl) and P5 along with 2 mg/ml gliadin that had been pretreated with pepsin, chymotrypsin, and trypsin. As seen in Fig. 4, P5 was efficiently eliminated by PEP, accumulation of the nontoxic peptide products PQPQP and QPQLPYP or QLPYPQP occurs as shown in Fig. 1. Thus, efficient cleavage of P5 was observed, even in the presence of a large number of smaller peptides.
gliadin fragments that might compete for the PEP active sites.

**A Model for Intestinal Content of Toxic Gliadin Peptides.** The intestinal content for P4 and P5 during intestinal transit was calculated using the in vivo data collected in this work (Fig. 5A). The model not only indicates the importance of intestinal digestion in these gastric and pancreatic resistant fragments but also highlights the relative resistances to proteolysis by the brush border enzymes. The model predicts that the concentration for the control peptide myoglobin should be reduced to <1% starting material within 100 cm (the human upper small intestine). However, P4 persists >375 cm (the human lower small intestine) and P5 declines only to ~5% after passing through the entire small intestine. In addition, data previously collected for P4 and P5 were used to determine the effect of transport across the epithelium on the concentration profile (Matysiak-Budnik et al., 2003). The overall effect on the luminal content was minimal, altering the average retention times of P4 and P5 by <7% (data not shown). On the other hand, copervation of 41.7 μg/ml recombinant PEP (~190 μU/μl) with P5 effectively reducing the luminal content to <1% with 75 cm, an 8-fold reduction in average retention time (Fig. 5B).

**Discussion**

Wheat-, rye-, and barley-derived peptides (named gliadins, secalins, and hordeins, respectively) are enriched in glutamine (35%) and proline (20%) residues (Wieser et al., 1983). Recent experiments have led to the hypothesis and verification that the high proline content renders portions of these proteins resistant to proteolysis by gastric, pancreatic, and intestinal brush-border enzymes (Hausch et al., 2002; Shan et al., 2002). Because Celiac Sprue has been linked to the presence of gliadin peptides in the intestinal lumen, defining a mechanism for further efficient processing of these gliadin peptides into smaller nontoxic fragments could possibly prevent the toxic interaction and prevent the intestinal damage in Celiac Sprue. Notably, PEP treatment of gliadin peptides has in fact been shown to reduce the immunostimulatory capacity of gliadin peptides (Shan et al., 2002). The work presented here quantifies the relative proteolysis resistance of several gliadin peptide segments and quantifies the in vivo role of prolyl endopeptidase as a potential enzymatic supplement to digestion of these toxic gliadin peptides. The luminal content model for gliadin peptide proteolysis illustrates the relative resistance of these gliadin peptides and the hydrolytic effects of PEP on luminal peptide content.

Incubating the panel of α- and γ-gliadin-derived peptide fragments with proteases that they are typically exposed to in the stomach and upper intestine (pepsin, trypsin, chymotrypsin, carboxypeptidase A, and elastase) revealed, with the exception of P6, that the peptides were almost completely resistant to proteolysis by these enzymes (>90% remaining after 4 h; Table 1). Certainly, there is a variable resistance of gliadin fragments to surface digestion, and some of the gliadin peptides are highly resistant to BBM processing. Because P6 is readily digested, it is unlikely to accumulate in the intestine to have a toxic effect due to macrophage stimulation (Tucková et al., 2000; Novák et al., 2002). The BBM was able to proteolyze some of the gliadin peptides, reducing them to <10% of the starting material, although this required 4 h of exposure under physiologically relevant conditions (Table 1). In particular, both P5 and P8 remained at ~70% of the starting material even after 4 h. These results indicate that the brush-border enzymes are capable of cleaving many potentially toxic gliadin peptides to smaller nontoxic fragments, but at relatively slow rates that can be expected to allow the parent peptide to reside for an extended period within the upper intestinal cavity to evoke a toxic response in the Celiac Sprue. Notably, the BBM action on P5, the 33-mer known to be highly immunogenic by its capacity to stimulate T cell replication, is minimal (Fig. 1). Only the C-terminal Phe residue is removed, leaving the still toxic 32-mer peptide as the product. (Although the in vitro BBM digestion data qualitatively represents the in vivo processes, the quantity of surface membrane peptidases provided by the intact intestine at the surface in vivo cannot be achieved even in high concentration BBM enzyme stocks. This obviates direct quantitative comparison of in vivo and in vitro brush-border studies.) The addition of PEP cleaves it further to 4- to 7-mers that are substrates for subsequent surface digestion by the BBM oligopeptidases. Whereas some peptides are
degraded into easily assimilated fragments, relatively long intermediates accumulate in the lumen in other instances (Fig. 1). These two points seem to indicate the possibility of two categories of digestive-resistant toxic peptides. The first category consists of highly resistant gliadin peptides such as P5 and P8; notably, P5 has already been shown to be a highly potent trigger of T cell proliferation from Celiac patients (Shan et al., 2002). The second category is composed of the less digestive-resistant, but still toxic, gliadin peptides that may be processed to nontoxic products in Celiac patients. Notably, the latter category of gliadin peptides may play a role only after the intestinal Celiac lesion has become well established with consequent reduction in brush-border enzyme expression (Mercer et al., 1990), thereby allowing the less resistant gliadin peptides to persist in the intestinal lumen.

At a low dose of PEP (25 μU/μl), the processing rates of the gliadin peptides were enhanced by ~50% (Fig. 2), but not to the high levels of digestion seen for the control peptides. When PEP concentration was increased to 190 μU/μl, in the in vivo perfusion experiments, P5 (33-mer) could be shown to be completely cleaved to small nontoxic products (Fig. 3). These dosing levels (1:50 to 1:5; PEP to peptide) compare favorably to dosing levels currently in use for treating lactose intolerance (1:100 to 1:10; lactase to lactose), indicating that these dosing levels are in a reasonable range. Also, despite the possible competitive effect of other gliadin peptide fragments that would be released by pancreatic protease action on gluten within the intestinal lumen, perfusion of gluten pretreated with pepsin and pancreatic proteases along with P5 had minimal effect on the efficient processing of the peptide to its nontoxic products (note that 85% of PEP’s P5 digestive capacity remained; Fig. 4). These data, along with digestive data for P5 with PEP alone, suggest that P5 is a high-affinity substrate for PEP (Shan et al., 2002). Currently, it is not known what quantities of intact peptide are required to initiate and maintain the Celiac inflammatory response. The compact nongliadin peptide GLGG is efficiently digested, and comparison of proteolysis for the gliadin peptides to those for GLGG, P10, and P9, may provide insight concerning the elimination of toxic gliadin in the intestine (Smith and Gray, 1977). The in vivo rate data indicate that toxic gliadin peptide digestion is an order of magnitude slower than that for GLGG and is reduced by >50% compared with the digestion of the nongliadin peptides. It has been suggested that PEP is capable of accelerating the proteolysis of resistant gliadin peptides. In the work presented here, PEP-supplemented digestion of P5 and P8 showed significant increases in proteolysis rates in both in vitro and in vivo assays. Although there are some peptides, such as P7, that are resistant to PEP digestion, PEP addition was capable of decreasing the in vitro concentrations of the majority of gliadin peptides tested to below 10% of those persisting after maximal gastric and pancreatic protease action. Furthermore, in vivo PEP increases the rate of proteolysis by >50% (Table 1), additional PEP dosing indicates that luminal gliadin concentration of these peptides can be reduced or be potentially eliminated. The quantitative analysis here indicates that PEP has activity for large peptides, including the 33-mer (P5). However, this activity could in fact be enhanced by prior removal of terminal residues. As is evident from Fig. 1, PEP seems to completely process the 32-mer (indicated as B), whereas the relative effect on the intact 33-mer is relatively slower. This indicates that removal of the terminal Phe may be an enhancing factor in the digestion through PEP supplementation. Using the quantitative data collected here, profiles of the luminal content for P4 and P5 were calculated. These profiles clearly illustrate the proteolysis resistance of these gliadin peptides with the gliadin peptides persisting 4 to 8 times longer than a physiologically relevant peptide from myoglobin (Fig. 5A). The profile for luminal content was recalculated using previously collected data for flux of P4 and P5 across the epithelium, which determined a minimal effect on the luminal content (Matysiak-Budnik et al., 2003). Further support for the beneficial effects of PEP supplementation is gained by analyzing the PEP dosing experiment presented in a similar manner (Fig. 5B). These results clearly illustrate the dramatic effect of PEP on luminal peptides.

The work presented here has demonstrated the resistance of several immunogenic gliadin peptides to gastric, intestinal brush-border proteolysis. Furthermore, we have determined that there seem to be two levels of resistance, one at the gastric and luminal (pancreatic) protease level and one at the brush-border peptidase level. The in vivo rate data have shown that the reduced rate of proteolysis is not solely due to size and that PEP can function in concert with the brush-border peptidases by providing the initial key cleavages on a number of peptides so as to increase the overall rate of peptide processing. Dosing experiments have also confirmed that highly resistant peptides such as P5 can effectively be eliminated with PEP supplemented digestion with minimal accumulation of products. Perfusion of pepsin-, trypsin-, and chymotrypsin-digested gliadin with P5 has demonstrated that PEP is capable of digesting P5 even in the presence of smaller, more accessible targets that would be present under physiological conditions (Fig. 4). Digestion of P5 under these conditions is at least comparable with that for the other gliadin digestive resistant fragments released after completed processing of gluten by pepsin, trypsin, and chymotrypsin, further suggesting that PEP has appreciable potential as a digestive supplement. Luminal profiles of gliadin content illustrate the relative proteolysis resistance of these gliadin peptides compared with physiologically relevant myoglobin peptide and that flux across epithelial cells only plays a minor role in determining luminal gliadin content (Fig. 5A). Furthermore, the abundance of toxic peptides calculated from the PEP dosing experiments establishes a dramatic effect of PEP on luminal concentration with a 3-fold increase in rate, resulting in an 8-fold reduction in average retention time (Fig. 5B).

These experiments have further advanced the hypothesis of PEP-enhanced digestion of gliadin as a means of therapeutic intervention for Celiac Sprue. To continue making progress in this area, it is important to continue whole gliadin proteolysis studies, to determine other potentially resistant fragments. This should allow the identification, quantification, and the stimulatory capacity of PEP-resistant peptides to be defined. Given the importance of understanding the digestion of these gliadin peptides and of determining the means to detoxify these peptides, it is important to elucidate the in vivo situation of a complex organ such as the intestine. Using the rat intestinal perfusion setup as our model system of digestion, more physiologically relevant experiments can now be explored with pepsin-digested gluten
in the intact duodenum where luminal protein digestion is very active. Although PEP cannot be expected to cleave intact gluten, it does cleave the gliadin peptides that are released by pancreatic enzyme digestion. Perfusion through the duodenum will determine the stability of PEP to endogenous pancreatic enzymes and determine whether PEP can effectively eliminate the toxic gliadin peptides, thereby protecting the upper regions of the small intestine. Perfusion experiments will also allow the continued study of PEP as a potential detoxifying agent, providing insight into the key events in the digestion and uptake of these gliadin peptides in order to prevent the cascade of events that follows in Celiac Sprue.

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References


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