

Intestinal digestive resistance of immunodominant gliadin peptides

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Hausch, Felix, Lu Shan, Nilda A. Santiago, Gary M. Gray, and Chaitan Khosla. Intestinal digestive resistance of immunodominant gliadin peptides. *Am J Physiol Gastrointest Liver Physiol* 283: G996–G1003, 2002. First published June 5, 2002; 10.1152/ajpgi.00136.2002.—Two recently identified immunodominant epitopes from α -gliadin account for most of the stimulatory activity of dietary gluten on intestinal and peripheral T lymphocytes in patients with celiac sprue. The proteolytic kinetics of peptides containing these epitopes were analyzed in vitro using soluble proteases from bovine and porcine pancreas and brush-border membrane vesicles from adult rat intestine. We showed that these proline-glutamine-rich epitopes are exceptionally resistant to enzymatic processing. Moreover, as estimated from the residual peptide structure and confirmed by exogenous peptidase supplementation, dipeptidyl peptidase IV and dipeptidyl carboxypeptidase I were identified as the rate-limiting enzymes in the digestive breakdown of these peptides. A similar conclusion also emerged from analogous studies with brush-border membrane from a human intestinal biopsy. Supplementation of rat brush-border membrane with trace quantities of a bacterial prolyl endopeptidase led to the rapid destruction of the immunodominant epitopes in these peptides. These results suggest a possible enzyme therapy strategy for celiac sprue, for which the only current therapeutic option is strict exclusion of gluten-containing food.

celiac sprue; brush-border membrane; peptidase; prolyl endopeptidase; kinetics

CELIAIC SPRUE IS A DISORDER of the small intestine induced by dietary exposure to gliadins¹ from wheat and to related proteins from barley, rye, and possibly other common food grains (23, 20). Ingestion of such proteins produces flattening of the normally luxurious, rug-like, epithelial lining of the small intestine known to be responsible for efficient and extensive terminal digestion of peptides and other nutrients. The disease has a prevalence of ~1:200 in most of the world's population

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¹Gliadins are the alcohol-soluble protein fraction of wheat gluten. They are typically rich in glutamine and proline, particularly in the NH₂-terminal part. For example, the first 100 amino acids of α - and γ -gliadins contain ~35 and 20% of glutamine and proline residues, respectively. For a more detailed analysis of gliadin chemistry, see review by Wieser (27).

groups, and evidence is mounting that intestinal damage is caused by interactions between specific gliadin oligopeptides and the human leukocyte antigen (HLA)-DQ2 (or DQ8) molecule, which in turn induce proliferation of T lymphocytes in the subepithelial layers (23). Clinical symptoms of celiac sprue include fatigue, chronic diarrhea, malabsorption of nutrients, weight loss, abdominal distension, and anemia, as well as a substantially enhanced risk for the development of osteoporosis and intestinal malignancies (lymphoma and carcinoma).

Recent reports have identified several short sequences from gliadins that activate gluten-specific T cells from celiac patients but not from control individuals in an HLA-DQ2-dependent manner. Of particular interest are the observations by independent laboratories that the majority of the intestinal T cell response to α -gliadin in adult patients is focused on two immunodominant epitopes, PFPQPQLPY and PQPQLPYPQ (2, 3). Designation of these epitopes as immunodominant is due to two factors. First, gluten-specific T cells from intestinal biopsies of nearly all patients respond to these epitopes. Second, when dietary gluten is administered to patients who have been on a gluten-free diet, T cells responsive to these immunodominant epitopes are detectable in peripheral blood significantly earlier than T cells that respond to other immunogenic gliadin epitopes. Although the mapping of epitopes of gluten proteins recognized by intestinal T cells is incomplete, these results suggest that a small number of related epitopes may be adequate to account for the primary inflammatory response in celiac sprue.

Regardless of the precise molecular mechanism of the toxic reaction to gluten, the primary event requires that epitope-bearing gliadin oligopeptides gain access to their antigen-binding sites, presumably within the lamina propria region interior to the relatively impermeable surface intestinal epithelial layer. Ordinarily, such oligopeptides, generated through the action of pancreatic proteases, are efficiently hydrolyzed into amino acids, di- or tripeptides by peptidases located in the brush-border membrane (BBM) of the intestinal

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enterocyte before they can be transported across the epithelial layer (12). However, gliadins and related proteins from rye and barley are unusually proline-rich (27), a residue inaccessible to most digestive proteases. Hence, we hypothesized that gliadin oligopeptides may be resistant to this normal digestive breakdown.

Normal assimilation of dietary proteins by the human gut can be dissected into three major phases: 1) initiation of proteolysis in the stomach by pepsin and highly efficient endo- and COOH-terminal cleavage in the upper small intestine cavity (duodenum) by secreted pancreatic proteases and carboxypeptidases; 2) further processing of the resulting oligopeptide fragments by exo- and endopeptidases anchored in the brush-border surface membrane of the upper small intestinal epithelium (jejunum); and 3) facilitated transport of the resulting amino acids, di- and tripeptides, across the epithelial cells into the lamina propria, from where these nutrients enter capillaries for distribution throughout the body (10, 12). Because most proteases and peptidases are unable to hydrolyze the substituted and conformationally constrained amide bond of proline residues, it might be expected that the abundance of proline residues in gliadins and related proteins from wheat, rye, and barley (20, 27) constitutes a major digestive obstacle for the enzymes involved in phases 1 and 2 above. This, in turn, would lead to an increased concentration of relatively stable gluten-derived oligopeptides in the gut, some of which would be expected to cross the intestinal barrier to gain access to the subepithelial lymphocytes.

With the use of soluble digestive proteases from bovine and porcine pancreas and BBM vesicles derived from the intestinal mucosa of normal adult rats, we show that immunodominant epitopes from gliadin (2, 3) are highly resistant to cleavage at the intestinal surface membrane, whereas they are unaffected by pancreatic endoproteases. In particular, two BBM peptidases, dipeptidyl peptidase IV (DPP IV) and dipeptidyl carboxypeptidase I (DCP I, also known as angiotensin-converting enzyme or peptidyl dipeptidase A), are rate limiting in their breakdown. These conclusions were also verified using BBMs derived from a human adult intestinal biopsy. Supplementation of the BBM with trace quantities of a bacterial prolyl endopeptidase (PEP) leads to rapid cleavage of these gliadin peptides to units much smaller than the binding site of the HLA molecules. This suggests a possible enzyme supplement therapy for celiac sprue, a lifelong disease in which the only current option is strict exclusion of gliadin-containing foods.

MATERIALS AND METHODS

The peptides QLQFPFQPQLPY, PQPQLPYPQPQLPY, and PQPQLPYPQPQLP were synthesized by the Stanford Protein and Nucleic Acid Facility and purified by preparative reversed-phase HPLC on a Beckman Ultrashpere C18 column (15 × 2.54 cm). HPLC purification of QLQFPFQPQLPY was accompanied by partial NH₂-terminal pyroglutamate formation leading to a pyroQLQFPFQPQLPY peak with a retention time of RT = 24.6 min. (This is a common phenom-

enon when peptides with NH₂-terminal glutamine residues are subjected to purification procedures; it is not a function of biological or enzymatic change.) Purified WQIQEQR was obtained from New England Peptides.

Enzyme substrates were purchased from Sigma (St. Louis, MO) except for Z-Gly-Pro-*p*-nitroanilide (Bachem, Switzerland) and captopril (ICN Biomedicals, OH). Angiotensin-converting enzyme (DCP I) from rabbit lung was provided by Cortex Biochem (San Leandro, CA). PEP from *Flavobacterium meningosepticum* (specific activity 28 nU/pg) was purchased from United States Biological (Swampscott, MA). A soluble form of DPP IV from *Aspergillus fumigatus* (expressed in *A. oryzae*) was obtained by Chemicon International (Temecula, CA). Trypsin (T-1250), chymotrypsin (C-4754), and carboxypeptidase A (C-0261) (all from bovine pancreas), elastase (E-1250) from porcine pancreas, bromelain (B-5144), papain (P-5306), and all other enzymes were obtained from Sigma.

Preparation of the BBM membranes. BBM fractions were partially purified from the small intestinal mucosa of adult female rats maintained on an ad libitum diet of wheat-based standard rodent chow (1). Total protein content was determined by a modified method of Lowry with bovine serum albumin as a standard (16). Alkaline phosphatase activity was determined with *p*-nitrophenyl phosphate (4). Sucrase activity was measured by using a coupled glucose assay (9). DPP IV, prolyl aminopeptidase, and aminopeptidase N (APN) were assayed continuously at 30°C in 0.1 M Tris·HCl, pH 8.0, containing 1 mM of the *p*-nitroanilides [extinction coefficient at 410 nm (ϵ_{410}) = 8,800 M⁻¹cm⁻¹] Gly-Pro-pNA, Pro-pNA or Leu-pNA, the latter in additional 1% dimethyl sulfoxide to improve solubility (15). DCP I activity was measured in a 100- μ l reaction as the release of hippuric acid from Hip-His-Leu (8). PEP activity was determined continuously with 0.4 mM Z-Gly-Pro-pNA in PBS:H₂O:dioxane (8:1.2:0.8) at 30°C (6). One unit is defined as the consumption of one micromole substrate per minute.

With the use of a similar procedure, a BBM fraction was also prepared from an intestinal biopsy sample derived from an adult human volunteer. Approval for two additional small pieces of tissue was obtained from the patient who required an esophagogastroduodenoscopy and biopsy for care. After isolation, the biopsy sample was immediately frozen in dry ice. The samples were thawed, weighed (~5 mg), and homogenized in 500 μ l ice-cold PBS buffer with the use of a Dounce homogenizer. The slow speed centrifugation step to remove cell debris was omitted to minimize loss of the scarce human material. The homogenate was centrifuged at 14,000 *g* for 50 min (4°C). Membrane-bound brush-border enzymes present predominantly in the pellet were resuspended in 500 μ l PBS and stored at -80°C in small aliquots. Total protein concentrations and specific enzyme activities of APN and DPP IV were measured as described above.

Liquid chromatography-coupled mass spectroscopy analysis of BBM and pancreatic digestion products. A standard 50- μ l digestion mixture contained 100 μ M of synthetic peptide, 10 μ M tryptophan, and Cbz-tryptophan as internal standards, resuspended BBM preparations with a final protein content of 27 ng/ μ l and/or exogenous proteases, as indicated, in PBS. After incubation at 37°C for the indicated time, the enzymes were inactivated by heating to 95°C for 3 min. The reaction mixtures were analyzed by liquid chromatography-coupled mass spectroscopy (LC-MS; SpectraSystem; ThermoFinnigan) using a C18 reversed-phase column (Vydac 218TP5215; 2.1 × 150 mm) with water:acetonitrile:formic acid (0.1%):trifluoroacetic acid (0.025%) as the mobile phase (flow: 0.2 ml/min) and a gradient of 10% acetonitrile for

3 min, 10–20% for 3 min, and 20–25% for 21 min followed by a 95% wash. Peptide fragments in the mass per charge ratio range of $m/z = 300$ –2,000 were detected by electrospray ionization mass spectroscopy using an LCQ ion trap (Thermo-Finnigan) and their identities were confirmed by tandem MS fragmentation patterns.

The MS intensities ($m/z = 300$ –2,000 g/mol) and UV_{280} absorbances of the parent peptides QLQPFPPQQLPY and PQPQLPYPQPQLPY were found to depend linearly on concentration in the range of 6–100 μ M. To estimate the relative abundance of the different intermediates, the reference peptides PQPQLPYPQPQLP, QLQPFPPQQLP, QPFPQPQLPY, and QPFPQPQLP were generated individually by limited proteolysis of the parent peptides with 10 μ g/ml carboxypeptidase A and/or 5.9 μ g/ml leucine aminopeptidase (L-5006) for 160 min at 37°C and analyzed by LC-MS as described above. The intrinsic mass intensity was identical (>95%) for PQPQLPYPQPQLP and QLQPFPPQQLP and approximately one-half for QPFPQPQLPY (>60%) and QPFPQPQLP (40%) compared with the starting material. The UV_{280} extinction coefficient of PQPQLPYPQPQLP was one-half of the starting material in accordance with the loss of one tyrosine residue.

RESULTS

Pancreatic protease digestion of gliadin peptides. We first examined the susceptibility of the peptides QLQPFPPQQLPY and PQPQLPYPQPQLPY to pancreatic digestion. These two sequences were derived from α_9 - and α_2 -gliadin, respectively, and have recently been shown to stimulate proliferation of T cells isolated from most celiac patients in an HLA-DQ2 restricted manner (3). Deletion analysis has led to the identification of the immunodominant epitopes PFPQPQLPY and PQPQLPYPQ in these peptides (3), also consistent with molecular recognition features of the HLA-DQ2 binding site (23).

PQPQLPYPQPQLPY was incubated with enzymes from bovine and porcine pancreas, and the digestion products were analyzed by LC-MS. Of the pancreatic endoproteases tested, only elastase at a high (non-physiological) concentration of 100 ng/ μ l was capable of partially hydrolyzing PQPQLPYPQPQLPY to 30% within 75 min. No proteolytic fragments were detected with trypsin or chymotrypsin under these conditions, and similar results were obtained with QLQPFPPQQLPY. This was expected, because these peptides do not contain any canonical trypsin or chymotrypsin cleavage sites. Carboxypeptidase A at a concentration of 100 ng/ μ l was capable of completely releasing the COOH-terminal tyrosine of PQPQLPYPQPQLPY within 75 min, but further proteolysis was efficiently blocked by the resulting COOH-terminal proline residue. These results suggest that the gliadin-derived epitopes are resistant to pancreatic endoproteolysis, whereas COOH-terminal processing might partially occur in the intestinal lumen.

Rat BBM preparation. Because pancreatic digestion products are further processed on the surface of the small intestine, we then investigated the pathways and associated kinetics of hydrolysis of immunodominant gliadin peptides treated with BBM preparations. Because the rodent is an excellent small animal model

for human intestinal structure and function (22), rat BBM was chosen as a suitable model system for these studies.

BBM fractions were prepared from rat small intestinal mucosa as described (1). With the use of standard assays, the specific activities of the known BBM peptidases were determined to be 127 μ U/ μ g for APN (EC 3.4.11.2), 60 μ U/ μ g for DPP IV (EC 3.4.14.5), and 41 μ U/ μ g for DCP I (EC 3.4.15.1). No prolyl aminopeptidase (EC 3.4.11.5) or PEP (EC 3.4.21.26) activity was detectable (<5 μ U/ μ g). Alkaline phosphatase and sucrase were used as control BBM enzymes with activities of 66 μ U/ μ g and 350 μ U/ μ g, respectively.

Although the levels of most intestinal peptidases in the rat are influenced by the dietary intake, the activity ratios relative to each other change only slightly. For example, DPP IV and DCP I are both upregulated by a high proline content (11, 24), but APN maintained its higher activity relative to DPP IV even when the animals were fed an unusually high proline-rich diet.

Rat BBM digestion of gliadin peptides. Proteolysis with rat BBM preparations was first investigated, using the peptide QLQPFPPQQLPY, a proteolytic product of α_9 -gliadin (3). This peptide was subjected to proteolysis by BBM, and the time course of digestion was followed by LC-MS analysis. Whereas the parent peptide QLQPFPPQQLPY disappeared with an apparent half-life of 35 min, several intermediates were observed to accumulate over prolonged periods of time (Fig. 1A). Indeed, their subsequent processing was substantially retarded (Fig. 1B). The identities of the major intermediates were confirmed by tandem MS and suggested an unusually high degree of stability of the PFPQPQLP sequence, a common motif in T cell-stimulating peptides (2, 3). Based on this data and the known amino acid preferences of the BBM peptidases (12), the digestive breakdown of QLQPFPPQQLPY was reconstructed, as shown in Fig. 1B, *inset*. The preferred pathway involves serial cleavage of the NH_2 -terminal glutamine and leucine residues by APN, followed by removal of the COOH-terminal tyrosine by carboxypeptidase P (CPP) and hydrolysis of the remaining NH_2 -terminal QP-dipeptide by DPP IV. As seen in Fig. 1B, the intermediate QPFPQPQLPY (formed by APN attack on the first two NH_2 -terminal residues) and its derivatives are increasingly resistant to further hydrolysis.

To estimate the relative resistance of QLQPFPPQQLPY toward BBM-mediated proteolysis, the proteolytic kinetics of a control gliadin peptide of comparable length, WQIPEQSR, were studied. This peptide is observed as a stable product after extensive treatment of recombinant α_2 -gliadin with a cocktail of pancreatic proteases (L. Shan et al., unpublished observation). It does not contain any epitopes known to be immunogenic in celiac sprue patients. In side-by-side experiments containing BBM peptidases, the stability of QLQPFPPQQLPY was fivefold higher than that of WQIPEQSR. More importantly, in contrast to QLQPFPPQQLPY, no intermediates were observed to accumulate in any reaction mixture containing WQIPEQSR

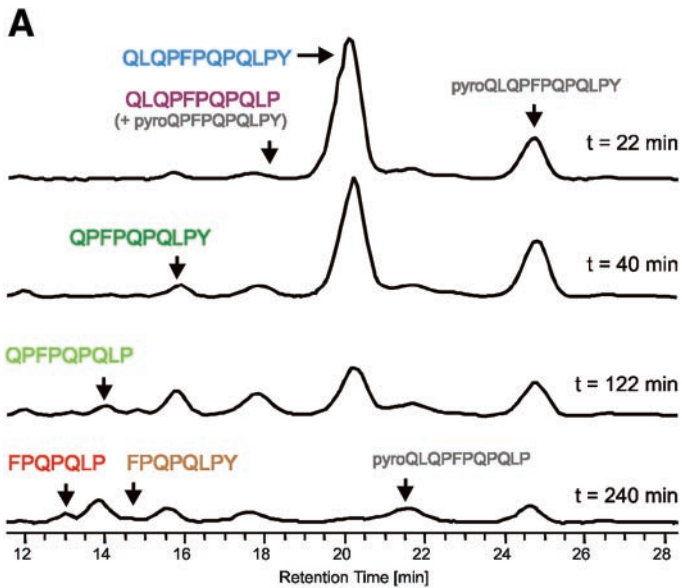
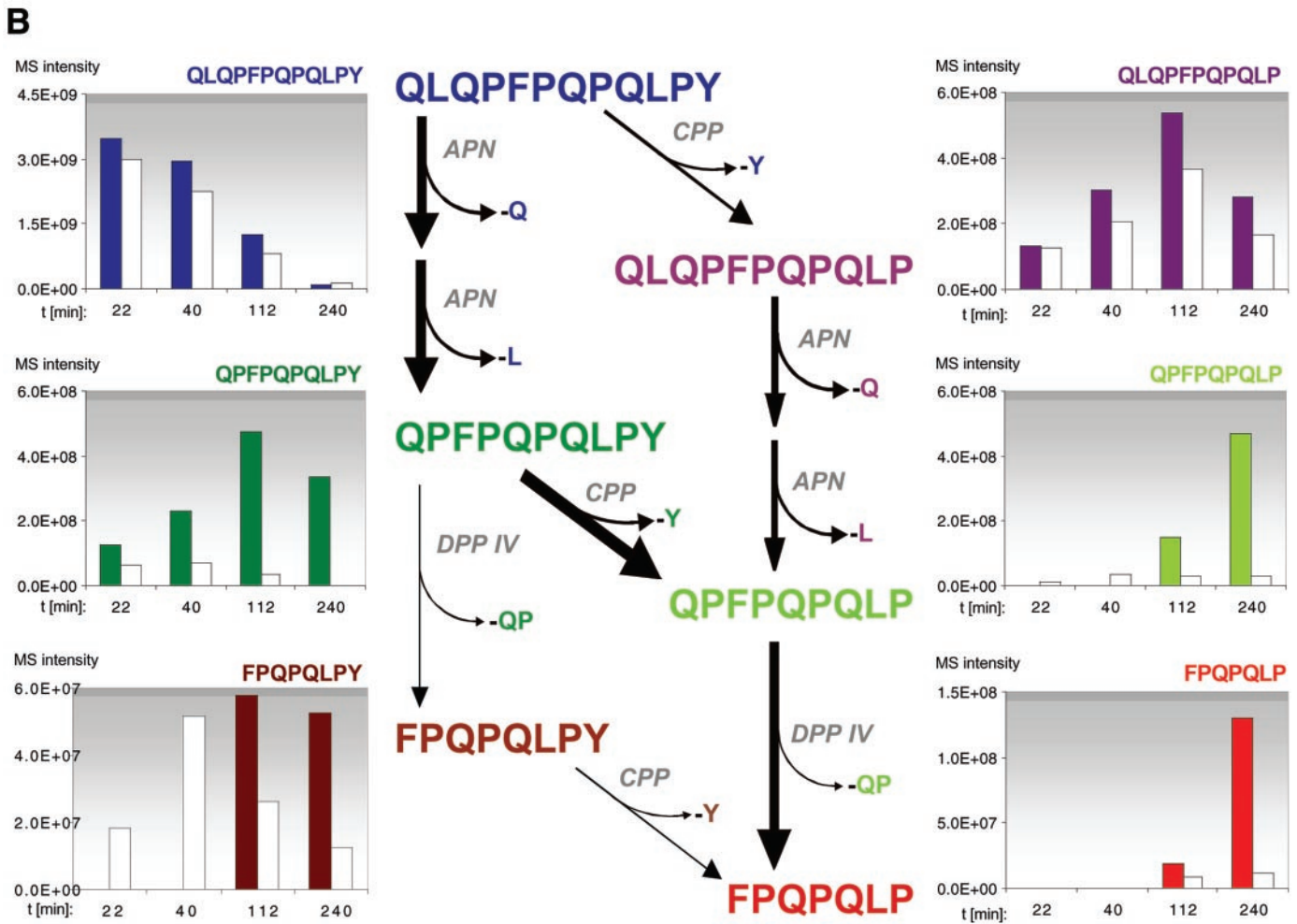


Fig. 1. Rat brush-border membrane (BBM) catalyzed digestion of the immunodominant gliadin peptide QLQPFQPQLPY. **A**: liquid chromatography-coupled mass spectroscopy (LC-MS) traces of QLQPFQPQLPY after digestion with 27 ng/ μ l rat BBM protein for the indicated time. Reaction products were separated by reversed-phase HPLC and detected by LC-MS (ion counts $m/z = 300-2,000$ g/mol). **B**: abundance of individual digestion products as a function of time. The peptide fragments in **A** were quantified by integrating the corresponding MS peak area ($m/z = 300-2,000$ g/mol). The resulting MS intensities are plotted as a function of digestion time (with BBM only, filled bars). The digestion experiment was repeated in the presence of exogenous dipeptidyl peptidase IV (DPP IV) from *Aspergillus fumigatus* (0.3 μ U DPP IV/ng BBM protein) and analyzed as above (open bars). The inserted scheme shows an interpretative diagram of the digestion pathways of QLQPFQPQLPY and its intermediates, the BBM peptidases involved in each step, and the amino acid residues that are released. The color code for labeling the peptides is the same as that used in **A**. The preferred breakdown pathway is indicated in bold arrows. APN, aminopeptidase N; CPP, carboxypeptidase P.



(data not shown; the highest observed concentration of the most abundant intermediate, IPEQSR, was <5% that of the parent peptide).

Because the three major intermediate products (QFPQPQLPY, QFPQPQLP, FPQPQLP) observed during BBM-mediated digestion of QLQFPQPQLPY are substrates for DPP IV, BBM treatment of QLQFPQPQLPY was repeated in the presence of a sixfold excess activity of exogenous fungal DPP IV. Whereas the relatively rapid decrease of the parent peptide and the intermediate levels of QLQFPQPQLP were largely unchanged, the accumulation of DPP IV substrates was entirely suppressed and complete digestion was observed within 4 h. (Fig. 1B, open bars).

To further investigate the rate-limiting steps in BBM-mediated digestion of gliadin peptides from the COOH-terminal end, another known peptide containing an immunodominant epitope present in wheat α -gliadin, PQPQLYPQPQLPY, was used (3). This peptide serves as a useful model for the analysis of COOH-terminal processing, because the NH₂-terminal end of this peptide can be considered proteolytically inaccessible due to minimal proline aminopeptidase activity in the BBM. As shown in Fig. 2, this peptide is even more stable than QLQFPQPQLPY. In particular, removal of the COOH-terminal tyrosine residue by CPP is the first event in its breakdown and more than four times slower than APN activity on QLQFPQPQLPY (Fig. 1B). The DCP I substrate PQPQLYPQPQLP emerges as a major intermediate after CPP catalysis and is highly resistant to further digestion, presumably due to the low level of endogenous DCP I activity naturally associated with the BBM. To confirm the role of DCP I as a rate-limiting enzyme in the COOH-terminal processing of immunodominant gliadin peptides, we supplemented the reaction mixtures with rabbit lung DCP I. Exogenous DCP I significantly reduced the accumulation of PQPQLYPQPQLP after overnight incubation in a dose-dependent manner (Fig. 2B). Conversely, the amount of accumulated PQPQLYPQPQLP increased more than twofold in the presence of 10 μ M of captopril, a DCP I-specific inhibitor, compared with unsupplemented BBM.

Together, the results reported in this section indicate that 1) gliadin peptides containing immunodominant epitopes are remarkably resistant to pancreatic and BBM-mediated proteolysis; 2) the proline-processing dipeptidases DPP IV and DCP I are essential for the NH₂- and COOH-terminal breakdown, respectively, of immunodominant epitopes at the rat intestinal BBM surface; and 3) these dipeptidases are rate limiting in the overall destruction of immunodominant epitopes by BBM. Presumably this explains the prolonged persistence of these proline-rich epitopes in the small intestine.

Validating the proteolytic resistance of immunodominant gliadin epitopes using human intestinal BBM preparations. To confirm that the above conclusions, derived from studies with rat BBM preparations, are also applicable to human intestinal digestion of immunodominant gliadin epitopes, BBM enzyme prepara-

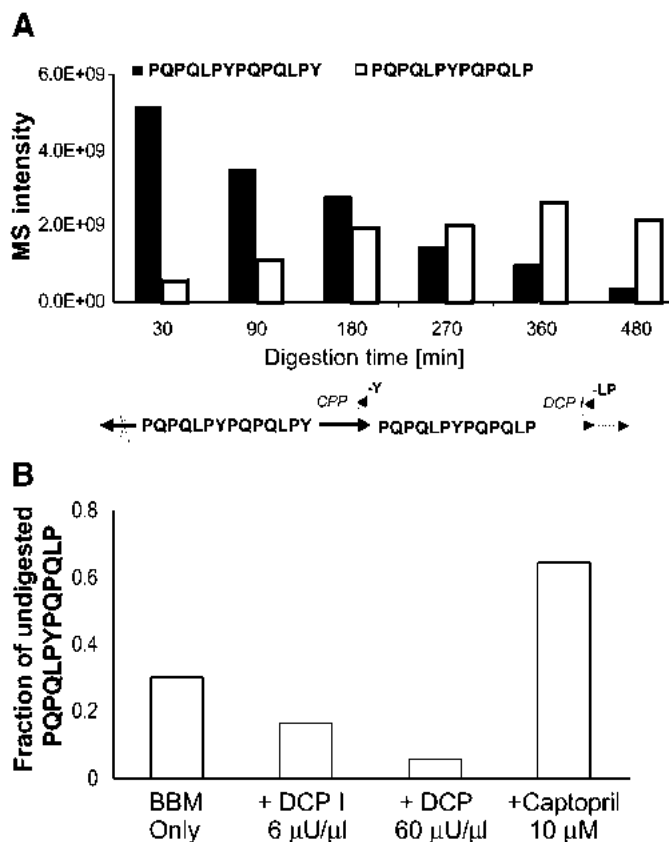


Fig. 2. COOH-terminal digestion of the immunodominant gliadin peptide PQPQLYPQPQLPY by rat BBM. A: PQPQLYPQPQLPY was digested by 27 ng/ μ l BBM protein preparations for the indicated time and analyzed by LC-MS as in Fig. 1A. The mass intensities ($m/z = 300$ – $2,000$ g/mol) of PQPQLYPQPQLP and the starting material were quantified as in 1B. All other intermediates were $\leq 1\%$. Bottom: schematic shows the proposed BBM digestion pathway of PQPQLYPQPQLPY with no observed NH₂-terminal processing (crossed arrow) and the removal of the COOH-terminal tyrosine by CPP (bold arrows). Further COOH-terminal processing by dipeptidyl carboxypeptidase I (DCP I) was too slow to permit analysis of the subsequent digestion steps (dotted arrows). B: influence of DCP I on COOH-terminal digestion. PQPQLYPQPQLPY was digested by BBM alone or with the addition of exogenous rabbit lung DCP I or captopril. After overnight incubation, the fraction of accumulated PQPQLYPQPQLP (compared to initial amounts of PQPQLYPQPQLPY at $t = 0$ min) was analyzed as in (A) with an acetonitrile gradient of 20–65% in 6–35 min.

tions were prepared from a distal duodenal biopsy sample from a patient who was found to have normal intestinal histology. The total protein concentration of this BBM preparation, determined by the Lowry method, was 1.0 μ g/ μ l. The specific activities of APN and DPP IV were 130 μ U/ μ g and 29 μ U/ μ g, respectively, and compared well with the specific activities of corresponding enzymes in rat BBM preparations (see above).

QLQFPQPQLPY and WQIPEQSR (100 μ M) were incubated with the human biopsy-derived BBM (final APN concentration 13 μ U/ μ l) at 37°C for varying time periods, and reaction products were analyzed with LC-MS. In summary, the results from these experiments were analogous to those obtained with rat BBM. For

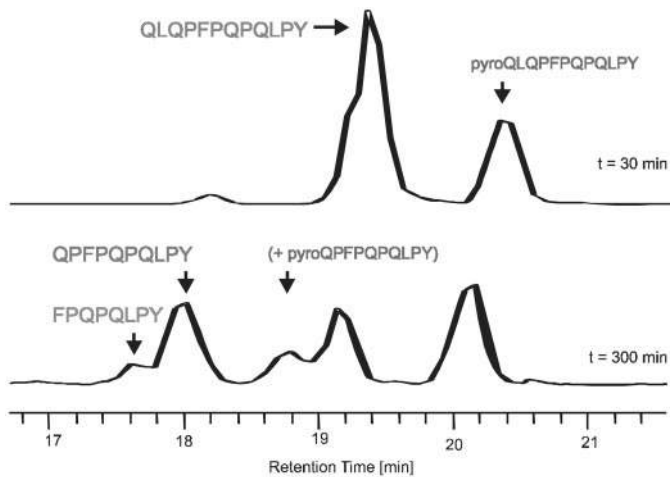


Fig. 3. Human BBM catalyzed digestion of the immunodominant gliadin peptide QLQPFPPQPQLPY. LC-MS traces of QLQPFPPQPQLPY after digestion with human BBM protein for 30 (*top trace*) and 300 (*bottom trace*) min. Reaction products were separated by reversed-phase HPLC and detected by MS. The ordinate scale in both traces is identical. A significant fraction of the hydrolyzed parent peptide accumulates as the DPP IV substrates QPFPPQPQLPY and FPQPQLPY (*bottom trace*).

example, the resistance of QLQPFPPQPQLPY toward exoproteolysis was underscored by the observation of significant quantities of the parent peptide at time points ≤ 300 min (Fig. 3). Moreover, the DPP IV substrates, QPFPPQPQLPY and FPQPQLPY, were abundant in the reaction mixture at 300 min, suggesting the rate-limiting nature of DPP IV in the proteolysis of immunodominant gliadin peptides. No product of either CPP or DCP I catalyzed breakdown of QLQPFPPQPQLPY was observed in significant quantities.

Accelerated breakdown of gliadin peptides by heterologous endopeptidases. Alerted by the high proline content as a hallmark of most immunogenic gliadin peptides, we speculated that a proline-specific endopeptidase would be ideal for the generation of new, free peptide termini. A literature search on available proteases led to the identification of PEP from *F. meningosepticum* that is specific for the COOH-terminal cleavage of prolines and readily available from recombinant sources (6, 28). Therefore, we digested the stable PQPQLPYPQPQLP intermediate with BBM in the presence of exogenous PEP. Figure 4 shows the dose-dependent acceleration of PQPQLPYPQPQLP digestion with increasing PEP concentration. As little as 3.5 pg PEP/27 ng BBM protein was sufficient to double the extent of proteolysis of this gliadin fragment compared with incubation with BBM alone. In comparison, other commonly used proteases like papain, bromelain, or porcine elastase were much less efficient, requiring 30-fold (papain) or 3,000-fold (bromelain, elastase) higher amounts of enzyme compared with PEP to give similar results. Their proteolysis was restricted to the cleavage of the Gln⁴-Leu⁵ and/or Gln¹¹-Leu¹² bonds. PEP had a preference for the Pro⁸-Gln⁹ and to a lesser extent the Pro⁶-Tyr⁷ bond of the PQPQLP[↓]YP[↓]QPQLP peptide. A similar preferential cleavage was found for

QLQPFPP[↓]QPQLPY. This is in agreement with the preference of this PEP for a second proline in the S2' position (5). Based on this P[↓]XP motif and on our data, we can predict up to 16 new, major cleavage sites in the α_2 -gliadin sequence [a major source of immunodominant epitopes identified thus far (3)] on PEP treatment. All of these cleavage sites are located in the critical NH₂-terminal part of α_2 -gliadin. The internal cleavage by PEP can be expected to generate additional (otherwise inaccessible) substrates for DPP IV and DCP I, thereby complementing the natural assimilation process of gliadins by the BBM. Thus the specificity of PEP is ideally suited for detoxification of persistent immunoreactive gliadin peptides in celiac sprue.

DISCUSSION

Understanding the molecular basis of gluten toxicity to celiac patients might allow for new therapeutic interventions in this wide-spread disease treatable so far only by a rigorous, life-long gluten abstention. Motivated by the exciting recent discovery of immunodominant epitopes in gliadin, we sought to investigate their susceptibility to proteolysis by pancreatic and intestinal endo- and exoproteases. From the above results, we propose that 1) both immunodominant epitopes are resistant to pancreatic endoproteases; 2) NH₂-terminal processing of epitopes, such as the one in the peptide QLQPFPPQPQLPY (epitope underlined) is retarded due to the rate-limiting activity of DPP IV; 3) epitopes such as those in PQPQLPYPQPQLPY can be considered resistant to COOH-terminal digestive breakdown because of the very low activity of DCP I; and 4) ingestion of gliadins such as α_2 -gliadin, where the above epitopes

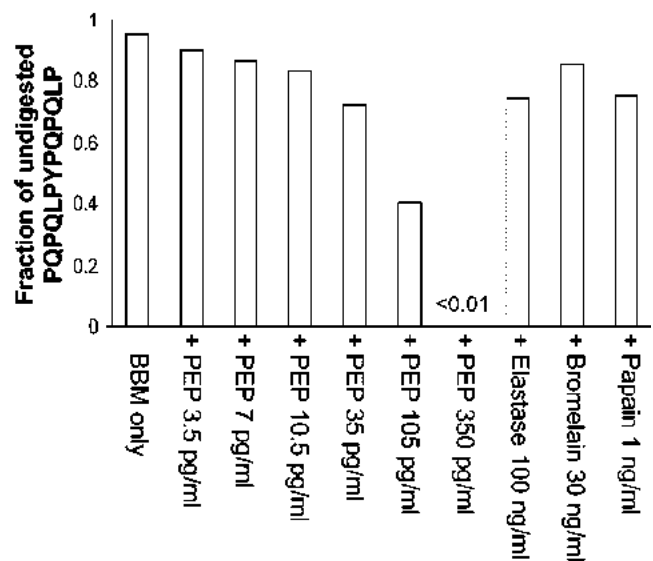


Fig. 4. Dose-dependent acceleration of BBM-mediated digestion by exogenous endopeptidases. The highly resistant peptide PQPQLPYPQPQLP (Fig. 2) was digested with 27 ng/ μ l BBMs, either alone, with increasing amounts of exogenous prolyl endopeptidase (PEP) from *Flavobacterium meningosepticum*, or with additional elastase, bromelain, or papain. After 1 h, the fraction of remaining PQPQLPYPQPQLP (as compared with the initial amount at $t = 0$ min) was analyzed by LC-MS and quantified as in Fig. 1.

overlap, is likely to result in a sustained high intestinal concentration of peptides capable of potent stimulation of intestinal T cells. Our results therefore provide a mechanistic explanation for the well-known but incompletely understood correlation between high proline content and celiac toxicity of storage proteins in various common cereals (20, 27).

In this report, we have analyzed the proteolytic fate of small peptides with boundaries at or close to the minimal T cell stimulating motifs (2, 3). However, given the scarcity of canonical pancreatic cleavage sites in the proline-rich gliadin sequence, immunogenic peptides generated by gastric-pancreatic digestion of gliadins *in vivo* can be expected to be substantially longer. If so, then repeated action of the rate-limiting BBM exopeptidases would be required for their detoxification, thereby further enhancing the persistence of immunogenic epitopes in the gut. A detailed analysis of the gastric-pancreatic digestion process of whole gliadin is, therefore, warranted to test this hypothesis.

Our analysis of the digestive pathways of immunogenic gliadin peptides also allowed us to ask whether enzymes capable of accelerating this exceptionally slow process could be therapeutically useful in the celiac diet. As shown in Figs. 1 and 2, addition of exogenous DPP IV and DCP I could compensate for the intrinsically slow proline processing by the BBM. However, both enzymes rely on efficient generation of free NH₂- and COOH termini by endoproteolytic cleavage. In contrast, a soluble bacterial PEP was extremely efficient at hydrolyzing the proline-rich gliadin fragments (Fig. 4) and, therefore, does not require extensive gliadin fragmentation by pancreatic proteases. Although PEP is expressed in human brain, lung, kidney, and intestine (14), no such activity has been reported in the BBM to our knowledge. Therefore, we suggest that supplementation of the celiac diet with bioavailable PEP (with or without DPP IV and DCP I) by virtue of facilitating gliadin peptide cleavage to nontoxic and/or digestible fragments may be useful in attenuating or perhaps even eliminating the inflammatory response to gluten. Such a strategy would be analogous to the enzyme therapy treatment in the case of lactose intolerance, where orally administered lactase is effective in cleaving and thereby detoxifying the lactose in milk products (7, 19).

PEPs have been isolated from several microorganisms, plants and animals and have been cloned from *A. hydrophila* (13), *Pyrococcus furiosus* (18), pig brain (17), and human T cells (21). These isozymes might constitute alternative detoxifying peptidases. Furthermore, the PEP used in this study is readily amenable to protein engineering by directed evolution (26). Thus optimization of PEP specificity toward immunogenic gliadin peptides seems possible once the determinants of toxicity become more clearly defined or new proline-rich epitopes are uncovered.

Finally, the finding that two intestinal surface exopeptidases (DPP IV and DCP I) are rate limiting in

the processing of immunogenic gliadin peptides raises the question of whether a subtle primary deficiency in one of these peptidases might enhance the probability of inducing the intestinal damage seen in celiac patients. Indeed, the importance of DPP IV in gliadin peptide digestion has been verified in DPP IV-deficient rats that lose weight when fed gliadin (25). A search for putative subtle deficiencies of the rate-limiting exopeptidases in patients with celiac sprue is warranted.

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