Rational Design of Combination Enzyme Therapy for Celiac Sprue

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Summary

Celiac sprue (also known as celiac disease) is an inheritable, gluten-induced enteropathy of the upper small intestine with an estimated prevalence of 0.5%–1% in most parts of the world. The ubiquitous nature of food gluten, coupled with inadequate labeling regulations in most countries, constantly poses a threat of disease exacerbation and relapse for patients. Here, we demonstrate that a two-enzyme cocktail comprised of a glutamine-specific cysteine protease (EP-B2) that functions under gastric conditions and a PEP, which acts in concert with pancreatic proteases under duodenal conditions, is a particularly potent candidate for celiac sprue therapy. At a gluten:EP-B2:PEP weight ratio of 75:3:1, grocery store gluten is fully detoxified within 10 min of simulated duodenal conditions, as judged by chromatographic analysis, biopsy-derived T cell proliferation assays, and a commercial anti-gluten antibody test.

Introduction

Celiac sprue is an inheritable enteropathy of the upper small intestine that is believed to afflict approximately 1:200 people in most parts of the world [1]. The disease is characterized by intestinal inflammation, villous atrophy, and crypt cell hyperplasia, resulting in a significant decrease in intestinal wall surface area [2]. This drastic reduction in surface area in turn triggers impaired nutrient absorption causing complications such as diarrhea, weight loss, anemia, and bone disorders, to name a few [3]. The principal environmental trigger has been identified as gluten from common food grains such as wheat, barley, and rye [2–4]. Strict exclusion of all forms of dietary gluten results in disease remission in most celiac sprue patients. However, the ubiquitous nature of gluten coupled with inadequate labeling regulations in most countries constantly poses a threat of relapse for patients, thereby lowering their quality of life. Nondietary therapies that allow celiac patients to safely incorporate low-to-moderate levels of gluten into their daily diet would therefore be of considerable benefit.

Wheat gluten is a complex mixture of polypeptides consisting of alcohol-soluble gliadins and alcohol-insoluble, disulfide-crosslinked glutenins. The primary structures of gluten proteins are dominated by proline (15%) and glutamine (35%) residues [5]. This unique structural feature, especially the high proline content, makes gluten unusually resistant to gastric and pancreatic proteases [6–9]. As a result, many long peptides (>20-mers) persist over a considerable length of the small intestinal lumen and are only partially hydrolyzed by the exopeptidases of the intestinal brush border membrane [10]. The inflammatory character of several of these metastable peptides has been extensively investigated [7, 9–11]. Perhaps most notably, some gliadin peptides are deamidated at selected glutamine residues by transglutaminase 2 (TG2), which further increases their affinity for disease-associated HLA DQ2 [11–15]. The resulting peptide-DQ2 complexes are potent triggers of inflammatory Th1 cell proliferation in the gut mucosa of celiac sprue patients [16]. Similarly, B cell epitopes in gluten are also comprised of proteolytically resistant proline- and glutamine-rich sequences [17]. Finally, at least one proteolytically resistant gliadin peptide is known to elicit an IL-15-mediated innate immune response in biopsies from celiac sprue patients [18, 19]. Thus, eliminating or reducing the luminal concentration of gluten-derived proline- and glutamine-rich immunotoxic peptides in the celiac small intestine can be expected to have significant therapeutic potential.

A variety of in vitro, in vivo (animal), and ex vivo (human) experimental approaches have shown that prolyl endopeptidases (PEPs) can readily cleave proline-rich gluten peptides, thereby reducing the antigenic burden of gluten [6–9, 20]. The potential to use PEPs as an oral enzyme treatment for celiac sprue is appealing because their pH-activity profile and substrate specificity complement mammalian pancreatic proteases. Each post-proline cleavage catalyzed by PEP on a metastable gluten peptide generates two shorter peptides, yielding one new amino and carboxyl terminus. In addition to directly reducing the antigenic burden of gluten, both of these outcomes yield peptides that are suitable substrates for further cleavage at the intestinal surface by constitutive brush-border aminopeptidases and carboxypeptidases. PEP can be readily formulated as an enteric-coated pill that protects the enzymatic activity from the harsh conditions of the stomach and rapidly releases its contents under simulated duodenal conditions [21].

A potential concern is that when administered as an active enzyme in the duodenum, PEP may not detoxify gluten fast enough to adequately protect a patient from multigram quantities of dietary gluten [22, 23]. Preclinical investigation of this potential limitation is hampered by the absence of a suitable animal model for HLA-dependent gluten enteropathy or sufficient insight into the time- and dose-dependence of the pathogenic
response to gluten in a patient. With regards to the latter option, seminal studies by Shiner and coworkers [24, 25] and by Marsh and coworkers [26, 27] demonstrated that: (1) histological and immunological changes are evident in the upper small intestine of some celiac sprue patients within 5–12 hr postgluten challenge, but not as evident in the upper small intestine of some celiac sprue that: (1) histological and immunological changes are divided by the proliferation in the absence of antigen. + SI > 2, ++ SI > 3.5, +++ SI > 5.0, +++ SI > 7.5.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>P26 TCL2</th>
<th>P28 TCL1</th>
<th>P28 TCL2</th>
<th>P34 TCL1</th>
<th>P34 TCL2</th>
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<tr>
<td>Glutenin PFSQQQPQV*</td>
<td>—</td>
<td>+</td>
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<td>Hor-9 analog PFOPQQPQF*</td>
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<td>26-mer FLQPQFPPQPOQPQYPQPOQPQPFPOQ*</td>
<td>++++</td>
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<td>++++</td>
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<tr>
<td>γ IV SQGPQQF[PQQPQ]*</td>
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<td>+</td>
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<tr>
<td>γ I POQSPQPOQO</td>
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<tr>
<td>28-mer PPPQQLPPQPOQQLPQPOQPQ*</td>
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The peptide sequence and common name are listed. The stimulation index (SI) is the T cell proliferative response in the presence of antigen divided by the proliferation in the absence of antigen. + SI > 2, ++ SI > 3.5, +++ SI > 5.0, +++ SI > 7.5.

Table 1. Characterization of Biopsy-Derived Polyclonal T Cell Lines with a Panel of Known Gluten Epitopes at 50 μM Peptide Concentrations

Results

Isolation and Characterization of Polyclonal T Cell Lines from Celiac Sprue Patients

Polyclonal gluten-responsive T cell lines derived from small intestinal biopsies of celiac sprue patients have been an invaluable tool for the identification of toxic gluten epitopes [12–14], as well as quantitative assessment of the gross toxicity of whole gluten digested with potential supplemental enzyme therapies [9]. In this study, we isolated six biopsy-derived, gluten-responsive T cell lines from four unrelated patients and characterized the epitope specificity of each line against a panel of known gluten epitopes (Table 1). Three of the cell lines from patients #26, #34, and #35 (named P28, P34, and P35 TCL1, respectively) were obtained by challenging biopsy samples with chymotrypsin- and human transglutaminase 2 (TG2)-treated gliadin. Two additional cell lines, also from patients #28 and #34 (P28 and P34 TCL2, respectively), were obtained by challenging the biopsies with a highly immunogenic 33-mer peptide from α2-gliadin (LQLOPFQPELPYQPELYQPELYQPELYQPFQ [7]. The sixth T cell line was derived from a biopsy sample from patient #26 (P26 TCL2) by first stimulating with chymotrypsin- and TG2-treated gliadin, followed by secondary stimulation with the synthetic 33-mer peptide in the presence of DQ2 homozygous VAVY cells (an EBV transformed B cell line) used as antigen presenting cells.

The epitope specificity of these T cell lines highlights the heterogeneous nature of this disease with respect to the spectrum of epitope recognition and magnitude of response. P28 and P34 TCL1 cell lines responded to most of the α- and γ-gliadin peptides tested and represented the cell lines with the broadest gluten responsive T cell repertoires. In contrast, the P26 TCL2 cell line responded nearly exclusively to the immunodominant α2 gliadin epitope. As shown in Table 1, the P26 TCL2 cell line also showed strong proliferation in the presence of the epitope-rich 26-mer peptide from γ-gliadin [10] at a concentration of 50 μM. However, at 5 μM peptide concentrations, the stimulation index of the 26-mer fell to 1.8, while the stimulation index resulting from α2 gliadin challenge remained at around 9.0 and fell below 2.0 only at the lowest concentration tested (0.05 μM). Further, an α2-specific T cell clone isolated from P26 TCL2 showed a similar response toward the 26-mer peptide, confirming
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a weak crossreactivity of some αII-specific T cell receptors with γ-gliadin epitopes (data not shown). Because relatively high concentrations of γ-gliadin epitopes were necessary to induce αII-specific T cell proliferation, P26 TCL2 is a useful probe for assessing the abundance of the αII epitope in digested whole gluten. It should be noted that the peptide panel used here is not an exhaustive list of gluten epitopes. There are other known [31] and putatively unknown epitopes [10] found in gluten that some of these polyclonal cell lines may recognize.

Comparing the Gluten-Detoxifying Ability of FM PEP and MX PEP

The polyclonal T cell lines generated and characterized as above were used to identify the most appropriate PEP for detoxifying gluten in the duodenum. In earlier studies, we compared the biochemical and pharmacological properties of several candidate PEPs [10, 21, 29]. Two PEPs from Flavobacterium meningosepticum (FM) and Myxococcus xanthus (MX) are among the most promising therapeutic candidates analyzed to date on account of their ability to proteolyze long gluten peptides, their preference for immunodominant T cell epitopes from α-gliadins, and their relative stability toward pancreatic protease attack. We therefore compared the kinetics of FM PEP- versus MX PEP-mediated detoxification of predigested gluten under simulated duodenal conditions. Food-grade gluten (12 mg/ml) was first treated with the gastric protease pepsin for 1 hr at pH 2.0. The pH was then raised to 6.0, and the pancreatic proteases trypsin, chymotrypsin, elastase, and carboxypeptidase A (TCEC) were added for 2 hr before being quenched by boiling. The resulting solution of proteolytically resistant peptides was then treated with FM or MX PEP for 30 min at gluten:PEP weight ratios of 375:1, 75:1, and 15:1. Four polyclonal T cell lines were used to assess the net antigenicity of these digests. The error bars represent the standard deviation of duplicate measurements.

Figure 1. Comparison of FM PEP versus MX PEP in Detoxification of Predigested Gluten at Three Different Doses

Gluten was digested with the gastric protease pepsin for 60 min at pH 2.0 followed by treatment with the pancreatic proteases trypsin, chymotrypsin, elastase, and carboxypeptidase A (TCEC) for 120 min at pH 6.0. The resultant mixture of digestively resistant gluten peptides was then treated with FM PEP or MX PEP for 30 min at gluten:PEP weight ratios of 375:1, 75:1, and 15:1. Four polyclonal T cell lines were used to assess the net antigenicity of these digests. The error bars represent the standard deviation of duplicate measurements.

As shown in Figure 1, neither PEP was able to completely detoxify gluten with respect to all T cell lines tested. Nevertheless, in general, FM PEP treatment resulted in lower T cell proliferation than MX PEP. Further, FM PEP was approximately 25 times faster at eliminating the immunodominant αII epitope than MX PEP as judged by the P26 TCL 2 T cell line. Therefore, FM PEP was selected for further evaluation as part of a two-enzyme cocktail.

EP-B2 Significantly Enhances Gluten Digestion under Simulated Gastric Conditions

In addition to proline residues, immunotoxic gluten peptides have high glutamine content (30%–35%) [5]. In a companion report in this issue of Chemistry & Biology [30], we have therefore expressed, purified, and biochemically characterized the zymogen form of a gluten-specifc cysteine protease from barley, EP-B2 [32]. In addition to its attractive sequence specificity, proEP-B2 is able to rapidly self-activate from its zymogen form under gastric conditions. The mature enzyme is highly resistant to pepsin proteolysis and has a desirable pH-activity profile (excellent activity in the pH 3–7.5 range) [30]. We therefore anticipated that a two-enzyme combination agent comprised of EP-B2 and PEP would accelerate gluten digestion in comparison to either enzyme alone, with EP-B2 proteolyzing dietary gluten into small peptides in the stomach, and the two-enzyme cocktail (in conjunction with pancreatic proteases) rapidly eliminating residual toxicity of gluten in the duodenum.

To investigate the effect of EP-B2 on gluten under gastric conditions, a high concentration of food-grade gluten (15 mg/ml; corresponding to a meal of ca. two slices of bread) was treated either with pepsin alone (25:1 gluten:pepsin) or pepsin supplemented with proEP-B2 (25:1 and 250:1 gluten:EP-B2). These experiments were performed at a pH of 4.5 to mimic the environment of a full stomach, although analogous experiments at pH 3 yielded similar results (data not shown). The reactions were sampled at 10, 30, 45, and 60 min and were quenched by boiling.

HPLC analysis (Figure 2) of the high-dose EP-B2 digests revealed a dramatic shift toward a shorter peptide fragment profile when pepsin was supplemented with EP-B2. The high concentration of long, minimally digested gluten peptides (eluting around 25 min), which persist in the presence of pepsin alone, is rapidly reduced within the first 10 min of EP-B2 addition.
The immunotoxicity of the reaction products generated by pepsin alone or pepsin plus EP-B2 was compared via T cell proliferation assays with the aforementioned gluten-responsive polyclonal T cell lines. After 10 min, both high- (25:1 gluten:EP-B2) and low- (250:1 gluten:EP-B2) dose EP-B2 digests showed a higher inflammatory capacity than gluten treated with pepsin alone (Figure 3). In contrast, after 60 min, gluten was partially detoxified by pepsin plus high-dose EP-B2, whereas the T cell reactivity of gluten treated with pepsin alone or pepsin plus low-dose EP-B2 continued to increase. Since some proteolysis is required to convert gluten polypeptides into shorter, bona fide antigenic peptides, these results suggest that EP-B2 can greatly accelerate the sluggish pepsin-catalyzed gastric digestion of gluten. However, notwithstanding the remarkable ability of this enzyme to simplify the texture and chemistry of gluten before food arrives in the primary affected organ, the data suggests that EP-B2 monotherapy may be insufficient for a majority of celiac sprue patients.

Synergistic Action of EP-B2 and FM PEP in a Potent Two-Enzyme Glutenase

To investigate the synergistic potential of the complementary chain-length tolerance, sequence specificity, and pH-activity profiles of EP-B2 and FM PEP, the kinetics of gluten detoxification under simulated duodenal conditions was investigated over a period of 60 min. For this, 15 mg/ml food-grade gluten was pretreated with pepsin alone or in combination with EP-B2 (25:1 gluten:EP-B2). The pH was then raised to 6.0, and physiological quantities of the pancreatic enzymes trypsin, chymotrypsin, elastase, and carboxypeptidase A (TCEC) with or without supplementation of FM PEP (75:1 gluten:FM PEP) were added. Samples collected after 0, 10, 30, and 60 min were analyzed via HPLC, proliferation assays with all six gluten-responsive polyclonal T cell lines, and a widely used antibody-based field test for measuring gluten content in everyday dietary products [33].

Remarkably, gluten treated with pepsin plus EP-B2 followed by pancreatic proteases plus FM PEP was completely nontoxic within 10 min exposure to the simulated duodenal environment (Figure 4A, see Supplemental Data for complete time-course data). To verify the complete detoxification observed with the two-enzyme combination, samples were titrated in T cell proliferation assays up to a high concentration of 1.5 mg/ml residual gluten. (The EC50 of gluten treated with pepsin plus pancreatic enzymes was <0.1 mg/ml for all lines tested.) Indeed, although residual antigenicity could be detected at the highest concentrations in samples exposed to either enzyme alone, the two-enzyme combination showed no T cell proliferative capacity at all concentrations tested (Figure 5). HPLC analysis of each sample confirmed the highly synergistic effect of EP-B2 and FM PEP on gluten digestion (Figure 4B, see Supplemental Data for HPLC data on dose optimization).
Finally, the toxicity of the gluten digests was assessed with a commercially available “gluten stick” test designed to recognize a common gluten pentapeptide sequence, QXP(W/F)P, in everyday food products [34]. Whereas the digest supplemented with FM PEP for 60 min tested positive for the presence of gluten at 200 ng/ml, the digest pretreated with EP-B2 for 60 min followed by pancreatic proteases for 10 min did not give a signal at all concentrations tested (up to 1,400,000 ng/ml) (Figure 6). Since this test can detect gluten down to 2 ppm [33], the glutenase-treated samples described here appear to have been completely detoxified as judged by both T cell and antibody reactivity.

Discussion

Celiac sprue is a widespread but severely underdiagnosed disease in which the major environmental (wheat gluten) and genetic (HLA DQ2) factors have both been identified [2, 4]. Yet, the only current treatment remains a strict life-long exclusion of wheat-, barley-, and rye-containing foods from the diet of celiac sprue patients. As a result of rapidly growing knowledge regarding the molecular basis of this disease [4], several therapeutic approaches can be envisioned [35]. Proteolytic detoxification of gluten in the patient’s gastrointestinal tract represents an especially attractive option because of both general safety considerations as well as the likelihood that if proven clinically effective, an oral glutenase agent would complement other therapies designed to render the patient less susceptible to gluten-mediated inflammation and enteropathy.

Guided by the primary structure of gluten, we have devised a two-enzyme glutenase to treat celiac sprue. By combining the glutamine-specific endoprotease EP-B2 with the well-characterized proline-specific endoprotease FM PEP, we have demonstrated rapid and complete abrogation of the immunotoxicity of grocery-store gluten, as measured by chromatographic analysis, patient-derived T cell assays, and a widely used antibody test for measuring gluten content in commercial food products. Not only did EP-B2 directly reduce the antigenic burden of gluten as indicated by T cell proliferation assays, but it also created better substrates for FM PEP, which prefers shorter peptides. Indeed, at a dose of 75:1 gluten:FM PEP, all gluten antigenicity was destroyed within 10 min by the addition of pancreatic proteases supplemented with FM PEP under simulated duodenal conditions following treatment with EP-B2 and pepsin under simulated gastric conditions.

Earlier studies by Marti et al. [9] demonstrated that FM PEP alone was able to reduce the T cell immunotoxicity of grocery store gluten that had been pretreated with pepsin followed by pancreatic proteases in 12 out of the 14 cell lines tested. These results were further validated by Pyle et al. [20], who showed that food-grade gluten that had been pretreated with FM PEP did not induce malabsorption in more than 50% of the patients who were adversely affected by vehicle-treated gluten. Thus, an enteric-coated form of FM PEP, which is protected from gastric inactivation and released in the duodenum [21], could provide clinical benefit by increasing the (yet to be determined) no-observed-adverse-effect level (NOAEL) of gluten in the majority of celiac sprue patients. However, the practical scope of FM PEP monotherapy will likely be limited by both the magnitude of this NOAEL increase as well as the percentage of patients that respond favorably to reasonable doses of the enzyme. In this study, we have therefore attempted to rationally design a glutenase therapy that could dramatically increase the NOAEL of gluten by combining two enzymes that complement each other with respect to sequence specificity and their ability to act in different locations of the gastrointestinal tract. Not only would this dual enzyme therapy allow celiac sprue patients to safely ingest larger quantities of gluten than FM PEP alone, but it may also be a more broadly applicable treatment for the disease. Although this therapy may not provide patients with a completely unrestricted diet, it should greatly enhance their dietary freedom.

Under kinetically demanding conditions where FM PEP monotherapy only had a modest effect in reducing...
the antigenicity of gluten, a combination of EP-B2 and FM PEP completely abolished T cell proliferation in all cell lines tested (Figure 4A). It should be noted that in these experiments, EP-B2 and FM PEP were coincubated with appropriate endogenous proteases (pepsin in the case of EP-B2, and trypsin, chymotrypsin, elastase and carboxypeptidase A in the case of EP-B2 and PEP). This mimics the in vivo environment by allowing for crossproteolysis of one protease by another. It should also be noted that full detoxification by the two-enzyme glutenase was observed without addition of intestinal brush border membrane enzymes [6, 9]. The presence of these enteric peptidases is likely to further accelerate the rate of gluten detoxification in vivo.

Although assays involving inflammatory T cells are the primary measures of gluten immunotoxicity in this study, recent studies have highlighted the potential role of other immune pathways in celiac sprue pathogenesis [18, 19, 36, 37]. While this report does not include experiments aimed at directly examining the effect of glutenase-treated gluten on the activation of these innate immune pathways, our HPLC (Figures 2 and 4B) and gluten antibody (Figure 6) results strongly suggest that the abundance of immunogenic gluten peptides of all lengths is greatly reduced by glutenase treatment. Further testing of this rationally designed glutenase in a clinical setting is therefore warranted.

Significance

Celiac sprue is a prevalent intestinal disorder estimated to affect 1:200 people in many populations around the world. The only current therapy is the absolute, life-long exclusion of wheat-, rye-, and barley-containing foods from the diet. A nondietary therapy that would allow patients to ingest moderate levels of these grains would therefore be of considerable benefit. We have devised and evaluated a two-enzyme glutenase consisting of the glutamine-specific endoprotease EP-B2 and the proline-specific endopeptidase FM PEP. EP-B2 acts on gluten under gastric conditions, whereas FM PEP eliminates the residual toxicity of gluten under duodenal conditions. Within 10 min of simulated duodenal conditions, the two-enzyme cocktail at
a dose of 75:3:1 gluten:EP-B2:PEP was able to completely detoxify gluten as judged by a variety of assays. Our results set the stage for clinical testing of this oral enzyme agent for a widespread unmet medical need.

Experimental Procedures

Isolation of Polyclonal T Cell Lines from Intestinal Biopsies Obtained from Celiac Sprue Patients

Small intestinal biopsies from celiac sprue patients were placed immediately into 1 ml HS T cell media (Iscove's Modified Dulbecco's Medium [Gibco, 12440-053] with 15% heat-inactivated human AB serum [Valley Biomedical, Inc.], 100 U/ml penicillin, and 100 µg/ml streptomycin [Gibco]) in a 1.5 ml Eppendorf tube. The tube was placed on ice and transported to the tissue culture laboratory within 20 min. The biopsy was then placed in 1 ml HS T cell media in a 24-well plate containing 1 mg/ml chymotrypsin-digested, tissue transglutaminase-deamidated gliadin (Sigma-Aldrich) (prepared as described in [11]) or 10 µg/ml deamidated 33-mer peptide (LQLQPF PQLQPFQQLPQQLPQQLPQQLPQQL) and incubated at 37ºC and 5% CO2 for approximately 20 hr. The biopsy was then extensively diced with a sterile scalpel, sheared by vigorous pipetting in HS T cell media, and mashed with a pipet tip against a 70 µm nylon filter (BD Falcon) in order to separate T cells from larger pieces of unbroken tissue. The recovered cells were counted and plated at 5–10 × 10⁴ biopsy-derived cells per well in the presence of 1–2 × 10⁵ γ-irradiated (4000 rads), autologous peripheral blood mononuclear cells.
Gluten digestion and analysis

(PBMcs) in a U-bottom 96-well plate (BD Falcon) containing 200 μl HS T cell media. The cultures were grown for 14 days with 50 U/ml IL-2 added on days 1, 3, 5, 8, and 11. For rapidly dividing lines, the cultures were split 1:2 when the media turned yellow.

For the second and subsequent rounds of stimulation, 1–5 × 10⁵ T cells and 25 × 10⁵ γ-irradiated (4000 rads) allogenic PBMcs were incubated in 25 ml FBS/HS T cell media (IMDM with 10% heat-inactivated fetal bovine serum [Atlanta Biologicals], 2% heat-inactivated human AB serum, 100 U/ml penicillin, and 100 μg/ml streptomycin) with 1 μg/ml PHA (Sigma-Aldrich) at 37°C and 5% CO₂ in a 25 cm² T-flask (Nunc) standing. These cultures were supplemented with 50 U/ml IL-2 on days 1, 3, 5, 8, and 11, and a media change was performed on day 5. For peptide-specific stimulation, the same protocol was used except 5 × 10⁵ ODQ2+ homologous B-LCL VAVY cells were incubated overnight with the peptide of interest (1 mg/ml gluten digest or 10 μg/ml peptide) on day −1 and added to the T cell/PBMc culture on day 0 in the absence of PHA.

**3H-Thymidine T Cell Proliferation Assay**

ODQ2+ homologous B-LCL VAVY cells were irradiated with 12,000 rads of γ-irradiation and incubated with protein digest or peptides overnight in FBS/HS T cell media at 2 × 10⁵ cells/ml in 96-well flat bottom plates. The next day, the volume was doubled to give 1 × 10⁶ VAVY cells/ml, and 50 μl was placed into a U-bottom 96-well plate. The frozen T cell lines were thawed and allowed to incubate in FBS/HS T cell media for 1–2 hr. Then, the T cell lines were resuspended to a concentration of 1 × 10⁶ cells/ml, and 50 μl was added to each well of the U-bottom 96-well plate to give 5 × 10⁵ VAVY cells and 5 × 10⁵ T cells per well in 100 μl final volume. These cells were incubated at 37°C and 5% CO₂ for 20 hr at which point 0.5 μCi/ml of [methyl-3H]-Thymidine (Amersham, TRK120) was added. The cells were incubated for an additional 12–14 hr, and incorporated thymidine was collected on a filter mat (Wallac, 1205-401) with a Tomtec centrifuge for 5 min. After this incubation, the specified amount of PEP enzyme was added and the final mixture was incubated at 37°C for 5, 10, 15, or 30 min. Both enzymes were concentrated prior to use to achieve greater than 320 activity units/ml. The PTECC-digested gluten (200 μl) and a specified amount of 25 mM Na₂HPO₄ buffer (pH 6.0) were added to an Eppendorf tube and incubated at 37°C for approximately 5 min. After this incubation, the specified amount of PEP enzyme was added and the final mixture was incubated at 37°C for 5, 15, or 30 min. The 25 mM Na₂HPO₄ buffer amount was adjusted to maintain a constant final volume of 243 μl (and a gluten concentration of approximately 11.9 mg/ml). At the specified time, the samples were heat deactivated at 95°C for >5 min. The samples were centrifuged for 10 min at 13,400 × g and deamidated with recombinant TG2 prior to performing the T cell assay.

**Kinetic Analysis of EP-B2 Digestion of Gluten under Gastric Conditions**

Whole gluten (15 mg/ml) was digested under mock gastric conditions (pH 3.0 and 4.5 at 37°C) by 0.6 mg/ml pepsin or by 0.6 mg/ml pepsin supplemented with either 0.6 mg/ml proEP-B2 or 0.06 mg/ml proEP-B2. After 10, 30, 45, and 60 min, samples of the reaction were taken and boiled to quench. Half of the volume of these samples were used for HPLC analysis, while the other half was adjusted to pH 6.0, diluted to 5 mg/ml gluten, deamidated with TG2, and used in T cell proliferation assays.

**Digest and HPLC Analysis of Gluten Treated with EP-B2 and/or PEP**

Whole gluten (15 mg/ml) was digested under mock gastric conditions (50 mM sodium acetate buffer [pH 4.5] at 37°C) for 60 min by 0.6 mg/ml pepsin or by 0.6 mg/ml pepsin supplemented with 0.6 mg/ml proEP-B2. After 60 min, each of these mock gastric reactions was adjusted to pH 6.0 by addition of 50 mM Na₂HPO₄ (final concentration) and intestinal proteases (0.375 mg/ml trypsin, 0.375 mg/ml chymotrypsin, 0.075 mg/ml elastase, 0.075 mg/ml carboxypeptidase A), or intestinal proteases supplemented with 12.5 U/ml FM PEP were added, followed by further incubation under mock intestinal conditions (pH 6 at 37°C). At designated time points, reactions were stopped by boiling for 10 min. Quenched digests were clarified by centrifugation for 10 min at 9,300 × g.

A portion of each sample supernatant was diluted into HPLC solvent A (5% acetonitrile, 0.1% TFA) with an internal standard (TAME) and analyzed on HPLC with a C₁₈ reversed phase column by using a water-acetonitrile gradient containing 0.1% (v/v) TFA. A second portion of each sample supernatant from the quenched gluten digests was deamidated with recombinant TG2 and analyzed by T cell proliferation assays.

**EP-B2/FM PEP Dose-Ranging Experiments**

Gluten (15 mg/ml) was digested under mock gastric conditions in 20 mM acetate buffer (pH 4.5) for 10 or 30 min with pepsin (0.6 mg/ml) in the presence or absence of EP-B2 at 10:1, 30:1, or 100:1 gluten:EP-B2 weight ratios. The pH was then adjusted to 6.0 by adding Na₂HPO₄, and pancreatic enzymes (0.375 mg/ml trypsin and chymotrypsin, 0.075 mg/ml elastase, and 0.075 mg carboxypeptidase A) were added in the presence or absence of FM PEP at 24/1, 80/1, and 287.1 glutens/PEP weight ratios. At the indicated time points, a 200 μl aliquot was taken from the digestion and boiled to quench the enzymes. The samples were run on HPLC by using a C₁₈ reverse phase column with an internal standard (TAME).

**Gluten Antibody Test**

The assay was a modification of the instructions provided by the manufacturer (RIDA QUICK Gliadin, R-Biopharm). Gluten digestes were diluted to ten times the indicated concentrations in PBS, and 50 μl of the diluted samples were added to 450 μl of the dilution buffer provided by the manufacturer. The test sticks were dipped into the 500 μl solution and allowed to sit for 5 min before being removed and allowed to air dry.

**Peptide and Digest Deamidation**

Gluten peptides and digests were treated with 30 μg/ml recombinant human tissue transglutaminase in the presence of 2.5 mM CaCl₂ for 2 hr at 37°C. The reaction tubes were centrifuged for 2 min at 13,000 rpm to pellet insoluble material and frozen at −20°C until use in T cell proliferation assays.

**Production and Purification of Recombinant Enzymes**

FM PEP, MX PEP, EP-B2, and TG2

All recombinant enzymes were made and purified as previously described [29, 30, 38].

**Peptide Synthesis**

Peptides were synthesized by Boc/HBTU chemistry as previously described [39].

**Supplemental Data**

Supplemental Data include additional time- and dose-dependent gluten proteolysis data showing the benefit provided by EP-B2...
and FM PEP supplementation and are available at http://www.chembiol.com/cgi/content/full/13/6/649/DC1/.

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