Combination Enzyme Therapy for Gastric Digestion of Dietary Gluten in Patients With Celiac Sprue

JONATHAN GASS,* MICHAEL T. BETHUNE,† MATTHEW SIEGEL,* ANDREW SPENCER,§ and CHAITAN KHOSLA*‡,#

*Department of Chemical Engineering, Stanford University, Stanford, California; †Department of Biochemistry, Stanford University, Stanford, California; §Alvine Pharmaceuticals, Inc, Palo Alto, California; ‡Department of Chemistry, Stanford University, Stanford, California

Background & Aims: Celiac sprue is a multifactorial disease characterized by an inflammatory response to ingested gluten in the small intestine. Proteolytically resistant, proline- and glutamine-rich gluten peptides from wheat, rye, and barley persist in the intestinal lumen and elicit an immune response in genetically susceptible persons. We investigated a new combination enzyme product, consisting of a glutamine-specific endoprotease (EP-B2 from barley) and a prolyl endopeptidase (SC PEP from Sphingomonas capsulata), for its ability to digest gluten under gastric conditions. Methods: The ability of this combination enzyme to digest and detoxify whole-wheat bread gluten was investigated. In vitro and in vivo (rat) experimental systems were developed to simulate human gastric digestion, and the resulting material was analyzed by high-performance liquid chromatography, enzyme-linked immunoabsorbent assay, and patient-derived T-cell proliferation assays. Results: The analysis revealed that EP-B2 extensively proteolyzes complex gluten proteins in bread, whereas SC PEP rapidly detoxifies the residual oligopeptide products of EP-B2 digestion. In vitro dose variation data suggests that an approximate 1:1 weight ratio of the 2 enzymes should maximize their synergistic potential. The efficacy of this 2-enzyme glutenate was verified in a rat model of gastric gluten digestion. Conclusions: By combining 2 enzymes with gastric activity and complementary substrate specificity, it should be possible to increase the safe threshold of ingested gluten, thereby ameliorating the burden of a highly restricted diet for patients with celiac sprue.

Celiac sprue is an inherited disorder of the small intestine that affects ≤1% of the population.1,2 This disease is characterized by an inflammatory response to ingested wheat gluten and similar rye and barley proteins, leading to small intestinal mucosal injury and nutrient malabsorption. If untreated, celiac sprue is associated with anemia, infertility, osteoporosis, and intestinal lymphoma.3–6 A strict, lifelong gluten-free diet is the only accepted treatment. Complete exclusion of dietary gluten is difficult due to the ubiquitous nature of this protein, cross-contamination of foods, inadequate food labeling regulations, and social constraints.7

Among the main dietary proteins, gluten is unique in that it contains approximately 15% proline and 35% glutamine residues.8 The high proline and glutamine content prevents thorough proteolysis by gastric and pancreatic enzymes, resulting in the buildup of long oligopeptides in the small intestine that are toxic to patients with celiac sprue. Therefore, proline- and glutamine-specific endoproteases, often referred to as glutenases, are proposed as therapeutic agents for celiac sprue on account of their ability to detoxify proteolytically resistant gluten epitopes.9–12 A combination of a gastrically active, glutamine-specific endoprotease (EP-B2; a cysteine endopeptidase from germinating barley seeds) and a duodenally active, proline-specific endopeptidase (prolyl endopeptidase; PEP) was shown to rapidly detoxify gluten under simulated gastrointestinal conditions.13

Here, we evaluate a new combination therapy, consisting of 2 gastrically active enzymes that detoxify gluten before its release in the small intestine. The main advantages of gastric glutenas are the following: (1) gluten can be fully detoxified before its arrival in the affected organ, (2) the enzyme(s) do not require formulation via enteric coating, and (3) enzyme stability in the presence of bile acids is not an important concern.14 Our objective was to identify a PEP capable of acting on gluten peptides in concert with barley EP-B2 in the stomach. Specifically, the PEP from Sphingomonas capsulata (SC PEP) was evaluated, because it has an extended pH profile compared with other PEPs.11,15,16

To simulate realistic gluten-containing diets, we selected whole-wheat bread as a test article for this study. All earlier studies were performed on synthetic gluten oligopeptides, recombinant gliadin proteins, or uncooked gluten. This gluten presentation is not fully rep-
representative of an actual meal. Gluten structure, in particular its level of disulfide bonding and its accessibility to proteolytic digestion, changes during the bread-making process. Therefore, the efficacy of the EP-B2 and SC PEP combination was first evaluated via in vitro digestion of whole-wheat bread and then confirmed by in vivo studies in rats.

Materials and Methods

Materials

Whole-wheat bread (Alvarado St Sprouted Whole-Wheat Bread) was from Alvarado St Bakery (Rohnert Park, CA). Pepsin was obtained from American Laboratories (Omaha, NE). Trypsin (from bovine pancreas, T4665), α-chymotrypsin (type II from bovine pancreas, C4129), elastase (from porcine pancreas, E7885), and carboxypeptidase A (type II from bovine pancreas, C-0386) were from Sigma (St. Louis, MO). Substrates for carboxypeptidase A (type II from bovine pancreas, C4129), elastase (from porcine pancreas, E7885), and carboxypeptidase A (type II from bovine pancreas, C-0386) were from Sigma (St. Louis, MO). Substrates for the chromogenic assays for PEP (Z-Gly-Pro-p-Nitroanilide) and EP-B2 (Z-Phe-Arg-pNA) were from Bachem (Torrance, CA). All materials used in the animal studies were food-grade or higher. Vancomycin was from Sigma. All other reagents were food or reagent grade.

EP-B2 and SC PEP Enzyme Manufacturing and Testing

EP-B2 was prepared in its zymogen form by existing protocol. Briefly, proEP-B2 was prepared in a 10-L fed-batch fermentation process that used a recombinant strain of *Escherichia coli*. The protein was purified from inclusion bodies by denaturing affinity chromatography, refolded, and concentrated in a similar manner as previously described. Details of the fermentation, purification, and refolding process were reported elsewhere. ProEP-B2 rapidly auto-activates under acidic conditions (pH < 5), with full activation occurring within 15 minutes at pH 4.5. As further evidence for gastric activation of proEP-B2, earlier studies showed that coadministration of proEP-B2 and gluten to rats facilitates gluten digestion while the food is still in the stomach.

SC PEP was prepared as described previously. EP-B2 concentration was between 5.8 and 15.5 mg/mL in 100 mmol/L Tris-Cl, 5 mmol/L EDTA, 2 mmol/L β-mercaptoethanol, 15% sucrose, pH 8, with specific activity ranging between 800 and 5000 U/mg. SC PEP was prepared in 20 mmol/L sodium phosphate buffer, pH 7, or phosphate-buffered saline, pH 7.4, at a concentration between 60 and 90 mg/mL and specific activity of 15–20 U/mg. Enzyme activity assays were performed as described previously.

In Vitro Whole-Wheat Bread Digestion

To evaluate the efficacy of alternative glutenases, an in vitro experimental protocol was developed to mimic the ingestion and digestion of whole-wheat bread from a grocery store. Alvarado St Sprouted Whole-Wheat Bread was selected because of its high protein level (label claim of 4 g protein for 38-g slice). A portion of a bread slice (typically 1 g) was presoaked with specified amounts of EP-B2 and SC PEP solutions, formulated in their respective buffers. Additional EP-B2 refolding buffer (100 mmol/L Tris-Cl, 5 mmol/L EDTA, 2 mmol/L β-mercaptoethanol, 15% sucrose, pH 8) was added to the bread so that 888 μL total liquid was added to 1 g bread. This additional buffer was added to allow for variation in the EP-B2 enzyme dosage. The bread was divided into 6 pieces.

To initiate the in vitro digestion protocol, the presoaked bread pieces were added to a 0.01 N HCl solution (pH 2, preincubated at 37°C) containing 0.6 mg pepsin/mL. Approximately 6.67 mL 0.01 HCl solution was added to 1 g bread (starting weight before any liquid addition) to achieve a final protein concentration of approximately 15 mg/mL in the suspension. The bread pieces were added over 15 minutes (at 3-minute intervals) and, after addition of each piece, the mixture was manually agitated with a spatula. The pH was approximately 4.5 at the end of the ingestion phase.

The simulated gastric digestion phase was considered to start on addition of the last bread piece to the 0.01 N HCl solution. The material was incubated at 37°C for various times (typically, 10 minutes to mimic short gastric digestion or 60 minutes to mimic extended gastric digestion). Samples (500 μL) were taken at 0, 10, 30, and 60 minutes and immediately heated at >95°C for at least 5 minutes to inactivate the enzymes. The mixture was manually agitated with a spatula before each sampling event.

In experiments in which duodenal digestion was simulated, at the end of the gastric phase, the pH was adjusted to 6.0 by the addition of sodium phosphate (15 mg for a 1-g bread digest) and 1 M HCl or 1 M NaOH or both. Pancreatic enzymes (trypsin, chymotrypsin, elastase, and carboxypeptidase A), prepared in ~50 mg/mL stock solutions, were added to yield the following final concentrations: 0.375 mg trypsin/mL, 0.375 mg chymotrypsin/mL, 0.075 mg elastase/mL, and 0.075 mg carboxypeptidase A/mL. The simulated duodenal digestions were performed with either trypsin and chymotrypsin only on all 4 pancreatic enzymes. The precise cocktail of pancreatic enzymes used in a given experiment is specified. The use of different pancreatic enzyme cocktails does not significantly affect the results or conclusions because elastase and carboxypeptidase A had minimal incremental effect on gluten digestion, which is consistent with previous work.

The final solution was then incubated at 37°C for up to 30 minutes. Samples (500–1000 μL) were withdrawn at 10 and 30 minutes and heat-treated as described previously.

In Vivo Whole-Wheat Bread Experiments in Rats

An existing animal experimental protocol for monitoring gluten digestion in rats was modified to evaluate the effect of EP-B2 + SC PEP on whole-wheat
breads (protocol was approved by animal welfare committee). Briefly, rats were acclimated to eat bread over the course of 2 days. The rats were fasted for a minimum of 12 hours (maximum of 24 hours) before the start of the experiment. They were then fed 4 g bread (starting weight) that had been pre-soaked with specified levels of EP-B2 (30 U protein/mg), EP-B2 (30 U protein/mg) + SC PEP (1.67 U protein/mg), or buffer alone (100 mmol/L Tris-Cl, 5 mmol/L EDTA, 2 mmol/L β-mercaptoethanol, 15% sucrose, pH 8). The rats were allowed to eat and digest the bread meal for 120 minutes and were then euthanized by CO2 exposure. The gastric contents of the animals were collected in a 15-mL Falcon tube and immediately frozen using a dry ice or ethanol bath. Gastric material was analyzed by removing approximately 250 mg of the frozen sample, heating the 250 mg aliquot at >95°C for at least 10 minutes to inactivate all enzymes, and then centrifuging the material at 14,000 g for 10 minutes. The supernatant was collected and analyzed by high-performance liquid chromatography (HPLC) or T-cell proliferation assays.

Reverse-Phase HPLC

Samples from the in vitro whole-wheat bread digests or harvested from rat stomach were chromatographically separated on a 4.6 × 150 mm reverse-phase C18 protein and peptide column (Grace Vydac, Hesperia, CA) using Varian-Rayn Dynamax (Palo Alto, CA) SD-200 pumps (1 mL/minute), a Varian 340 UV detector set at 215 nm and a Varian Prostar 430 autosampler. Solvent A was water with 5.0% acetonitrile and 0.1% trifluoroacetic acid. Solvent B was acetonitrile with 5.0% water and 0.1% trifluoroacetic acid. Before injection, samples were centrifuged for 10 minutes at approximately 14,000 g and filtered through a 0.2-µM syringe filter.

Indirect Competitive ELISA for Gliadin Peptides

The relative amount of gliadin in each sample was determined by indirect competitive enzyme-linked immunoabsorbent assay (ELISA). To prepare the coating solution, 20 mg gliadin/mL (Sigma) was digested in 0.01 M HCl for 60 minutes at 37°C with 0.6 mg pepsin/mL. The reaction was then adjusted to pH 6.0 with Na2HPO4 and 0.375 mg trypsin/mL was added to further digest the gliadin for 120 minutes at 37°C. The reaction was quenched by boiling 10 minutes and frozen at −20°C until use.

On day 1 of the ELISA procedure, pepsin-trypsin-digested gliadin was diluted to 20 mg/mL in coating solution (50 mmol/L sodium carbonate/bicarbonate buffer, pH 9.6, 0.02% NaN3) and 200 mL/well was incubated overnight at 4°C in 96-well microtiter plates (Nunc Maxisorp; Nalge Nunc International, Rochester, NY). Samples were diluted 1:100 to 1:1,296,000 in Starting-Block T20 TBS blocking buffer (Pierce, Rockford, IL) and incubated overnight at 4°C with an equal volume of 5.1 mg rabbit polyclonal anti-gliadin antibody/mL (Sigma). The protein concentrations of the diluted samples ranged from ~3 pg/mL to 0.1 mg/mL. On day 2, antigen-coated plates were washed thrice with 1× phosphate-buffered saline, pH 7.4, 0.05% Tween-20 before blocking and between all subsequent steps. Plates were blocked with 200 mL blocking buffer/well for 2 hours at room temperature. Antibody or sample mixes were added to the wells in triplicate (200 mL/well) and incubated overnight at 4°C. On day 3, goat antirabbit IgG-alkaline phosphatase conjugate (Sigma) was diluted 1:250 in blocking buffer and 200 mL/well was incubated 3 hours at room temperature. Freshly prepared substrate solution (5 mg pNPP/mL, 50 mmol/L sodium carbonate/bicarbonate buffer, pH 9.8, 1 mmol/L MgCl2, 0.02% NaN3) was added (200 mL/well), and the absorbance at 405 nm was measured every 6 seconds for 5 minutes.

The initial rate (mA405/minute) in each well was determined from 31 data points. For each sample, the mean initial rate at each dilution was plotted against the corresponding dilution, yielding a hyperbolic curve. To determine the dilution required for half maximal reduction of initial rate for each sample (IC50), the inflection point of each curve was determined by using the HyperbolaGen model in the curve-fitting program Origin 6.0 (OriginLab Corporation, Northampton, MA). Values shown represent the mean of 3 separate experiments, each run in triplicate, on separate days unless otherwise indicated.

T-Cell Lines and 3H Thymidine T-Cell Proliferation Assay

The T-cell lines P28 TCL1 and P35 TCL1 were generated and characterized as previously described. T-cell proliferation assays were performed using DQ2 homozygous VAVY cells as described in Siegel et al except 1 µCi/well of [methyl-3H]-thymidine was pulsed for 24 hours before harvesting the cells. Samples were diluted to protein concentrations ranging from 0.03 to 1.8 mg/mL. Samples were analyzed in duplicate, and the counts per minute were measured by subtracting the counts per minute of VAVY cells alone. Blanks represent the counts per minute of T cells (minus the proliferation of VAVY cells) in the absence of any gluten sample. Stimulation index was determined by dividing the adjusted count per minute value by the blank value.

Results

In Vitro Digestion of Whole-Wheat Bread

To evaluate the efficacy of individual versus combination glutenase therapies, whole-wheat bread was digested in vitro according to protocols described in “Materials and Methods.” The digests were analyzed using HPLC analysis, indirect competitive ELISA, and T-cell proliferation assays. Note that in vitro digestion of bread
under simulated gastric conditions differs from that of uncooked whole gluten. In the case of whole gluten, most of the protein dissolves under simulated gastric conditions. In contrast, acid and pepsin alone are insufficient to dissolve the protein in bread, and pancreatic enzymes are required to fully solubilize the gluten protein (for further details, see supplemental Results; See Supplementary material online at www.gastrojournal.org).

**EP-B2 Digestion of Whole-Wheat Bread**

In a previous study, we have characterized in detail the reverse-phase HPLC profiles of whole gluten after treatment under simulated gastric and duodenal conditions. Under these analytical conditions, most immunotoxic peptides have retention times >12.5 minutes. For example, representative antigenic gluten oligopeptides composed of 9, 11, 12, 14, 21, and 28 residues elute at 12.5, 18.5, 21.5, 20, 22.5, and 22 minutes, respectively. A small fraction of the undigested gluten protein elutes at 25 minutes (the remainder being tightly bound to the guard column). The relatively broad 25-minute peak also includes other long gluten-derived peptides (>30 residues); for example, a highly immunogenic 33-mer peptide elutes at 25 minutes.

The glutenase activity of EP-B2 was evaluated at doses ranging from 10 to 200 U EP-B2/mg bread protein. Low levels of EP-B2 (10 U EP-B2/mg protein) were sufficient to dramatically change the gluten oligopeptide profile (Figure 1A and B). Notably, the abundance of late eluting peaks (23–25 minutes) reduced considerably, whereas the area under the curve from 12 to 23 minutes increased compared with the pepsin-only control. As the EP-B2/gluten ratio was increased, the area under the curve of all peaks eluting after 12 minutes reduced, whereas the early eluting peaks became more abundant. Interestingly, a steady decrease in the area under the curve corresponding to the gluten oligopeptide region (12–23 minutes) was observed up to the highest EP-B2 dose evaluated. In contrast, complete elimination of gluten metabolites eluting at 23–25 minutes was achieved at EP-B2 doses >30 U/mg protein, suggesting that EP-B2 preferentially digests longer gluten peptides. The addition of EP-B2 also caused an increase in the amount of soluble protein present in the digest (versus the pepsin-only control) (data not shown).

**EP-B2 and SC PEP Digestion of Whole-Wheat Bread**

To evaluate the synergistic potential of EP-B2 and SC PEP in the gastric environment, the combination
glutenase was compared with the individual enzymes (in the presence of pepsin) using the in vitro bread digestion protocol. As summarized above, small quantities of EP-B2 alone efficiently break down full-length gluten proteins into oligopeptides, although relatively higher concentrations of this enzyme are required to substantially reduce the abundance of gluten oligopeptides eluting between 12 and 23 minutes (Figures 1 and 2). In contrast, although SC PEP has high specificity for immunotoxic gluten epitopes, it has minimal ability to detoxify gluten (Figure 2), presumably because of its relatively low specificity for long peptide substrates. Therefore, for the combination glutenase agent to be effective, EP-B2 must rapidly cleave intact gluten proteins into oligopeptides, whereas SC PEP must efficiently attack internal proline residues in the resulting oligopeptides. In addition, the 2 enzymes must be reasonably stable in each other’s presence.

The results, presented in Figures 3–5 (HPLC and T-cell proliferation) and Table 1 (ELISA), highlight the synergistic capacity of EP-B2 and SC PEP to detoxify gluten. HPLC analysis of whole-wheat bread digested in vitro showed that SC PEP reduced the abundance of gluten oligopeptides eluting between 12 and 23 minutes that were generated by low (30 U) or high (200 U) EP-B2 doses (Figure 3A and B). Notably, the combination of EP-B2 (30 U) + SC PEP (0.5 U) was markedly more effective than high dose (200 U) EP-B2 alone.

T-cell proliferation assays, using 2 polyclonal cell lines derived from unrelated patients with celiac sprue, were also used to assess the residual gluten immunotoxicity of whole-wheat bread digested under simulated gastric conditions (Figures 4 and 5). At a low EP-B2 dose of 30 U/mg gluten, only a modest reduction in immunotoxicity was
observed compared with the pepsin-only control (Figure 4). However, when this EP-B2 dose was supplemented with SC PEP, the immunotoxicity of the resulting bread sample reduced markedly (Figure 4). At a higher EP-B2 dose of 200 U/mg gluten, this enzyme alone significantly reduced but did not eliminate bread gluten toxicity, whereas SC PEP supplementation did (Figure 5). These results lend further support to the hypothesis that EP-B2 and SC PEP should act synergistically on gluten in the stomach.

An indirect competitive ELISA, using a commercially available polyclonal antigliadin antibody (Sigma), was also used to compare the relative abundance of intact gliadin sequences in bread samples that had been digested by alternative enzyme combinations. Because the epitope specificity of the antibody used in this assay is undefined, it was not possible to precisely correlate the intensity of the ELISA signal to the residual oligopeptide concentration. Notwithstanding this limitation, the assay verified the key conclusions derived from HPLC and T-cell studies summarized earlier. Specifically, as shown in Table 1, SC PEP supplementation of low (10 U/mg protein) or high (100 U/mg protein) EP-B2 doses reduced the immunoreactivity of the corresponding bread digest.

Finally, the stability of the 2 glutenases investigated in this report was estimated in each other’s presence. At a dose ratio of 30 U EP-B2 and 0.5 U SC PEP/mg bread protein, EP-B2 did not significantly alter SC PEP stability. Approximately 40%-50% of the initial SC PEP was retained after 60 minutes of simulated gastric digestion, independent of whether EP-B2 was present (data not shown). The activity loss of SC PEP was due to the exposure of this enzyme to low pH conditions, as also seen previously. Similarly, the presence of SC PEP did not affect EP-B2 activity.

**Identifying the Optimal Dose Ratio of EP-B2 and SC PEP**

To efficiently evaluate a combination glutenase in clinical trials, a fixed dose ratio of the 2 active ingredients must be specified. To determine this optimal dose ratio, each enzyme dose was systematically varied in simulated gastric digests of whole-wheat bread. The EP-B2/gluten ratio was varied between 18 U/mg protein and 147 U/mg protein (corresponding to a proEP-B2/gluten weight ratio of about 1:300 to 1:38). For each EP-B2/gluten ratio tested, the weight ratio of the 2 enzymes was varied between 0:1 (EP-B2 monotherapy) and 1:1 (equal dose of each enzyme on a weight basis). Two significant conclusions were drawn from this data. First, addition of SC PEP to proEP-B2 has a strong synergistic effect on gluten detoxification. For example, at a gluten/proEP-B2/SC PEP weight ratio of 75:1:1, gluten digestion was markedly superior to that observed at a gluten/proEP-B2 weight ratio of 38:1 (Figure 6). Indeed, more extensive gluten digestion was observed at a weight ratio of 75:1:0.25, as

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Recognition by antigliadin Abs*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin</td>
<td>+++</td>
</tr>
<tr>
<td>EP-B2 (10 U)</td>
<td>+++</td>
</tr>
<tr>
<td>EP-B2 (30 U)</td>
<td>+</td>
</tr>
<tr>
<td>EP-B2 (100 U)</td>
<td>+</td>
</tr>
<tr>
<td>EP-B2 (200 U)</td>
<td>—</td>
</tr>
<tr>
<td>EP-B2 (10 U) + SC PEP (1.67 U)</td>
<td>+</td>
</tr>
<tr>
<td>EP-B2 (30 U) + SC PEP (0.50 U)</td>
<td>+</td>
</tr>
<tr>
<td>EP-B2 (100 U) + SC PEP (0.50 U)</td>
<td>—</td>
</tr>
<tr>
<td>EP-B2 (200 U) + SC PEP (0.50 U)</td>
<td>—</td>
</tr>
</tbody>
</table>

NOTE. All digests were performed under simulated gastric conditions for 60 minutes. The results represent average values of 3 experimental runs (each performed in triplicate), except for the pepsin control and the EP-B2 (30 U) digests. For these conditions, multiple digests (n = 2 for pepsin; n = 3 for EP-B2 [30 U]) were tested.

Ab, antibody. Symbols used: +++ = 100% compared with pepsin control; 10% = +++ = 100%; 1% = < 10% = 1%. *A commercially available polyclonal antigliadin antibody (Sigma), whose epitope specificity is undefined, was used to estimate the abundance of residual gliadin oligopeptides in various samples by a competitive ELISA assay. The reactivity of this polyclonal antibody toward each sample was compared with the pepsin control.
compared with simply doubling the proEP-B2 dose. Second, although gluten is more thoroughly broken down as the SC PEP/proEP-B2 ratio is increased, the incremental benefit of SC PEP is nearly saturated at an SC PEP/proEP-B2 weight ratio of 1:1 (data not shown). We therefore anticipate that a fixed-dose ratio of approximately 1:1 (wt/wt) proEP-B2/SC PEP should be appropriate for clinical investigation.

**In vivo Activity of the EP-B2 + SC PEP Combination Glutenase**

The in vivo activity of the EP-B2 + SC PEP combination was evaluated using a rodent model of gastric digestion, which had been used earlier to assess the glutenase activity of EP-B2. In this experiment, the liquid enzyme formulations (or appropriate buffers as vehicle) were added onto whole-wheat bread and fed to fasted rats. At a specified time, the rats were killed. Their gastric contents were collected and analyzed by HPLC and T-cell proliferation assay. Three rats were tested under each of 3 experimental conditions (vehicle, EP-B2 [30 U], EP-B2 [30 U] + SC PEP [1.67 U]). A representative set of HPLC results, all of which were similar to analogous data obtained under in vitro conditions, is shown in Figure 7A. Addition of EP-B2 caused a marked increase in the area under the curve corresponding to gluten-derived oligopeptides (12–23 minutes of retention time), whereas inclusion of SC PEP resulted in a further increase in the early eluting peptides, accompanied by a reduction in the 12- to 23-min region of the HPLC trace. The EP-B2 + SC PEP therapy did not completely eliminate the 12- to 23-min gluten oligopeptide region, and the extent of gluten digestion was not as significant as shown with the in vitro model with analogous glutenase doses (data not shown).
shown). These results indicate that additional in vivo animal trials are warranted to analyze the actual capacity of these enzymes to eliminate immunogenic peptides before initiating clinical trials.

T-cell proliferation assays were also performed on the gastric samples isolated from rats using polyclonal cell lines derived from 2 unrelated patients with celiac sprue (Figure 7B and C). As was also observed in the context of simulated gastric digestions in vitro, proEP-B2 alone increases the inflammatory characteristics of bread gluten as compared with the glutenase-free control. This apparent increase in immunotoxicity is due to the inability of pepsin to sufficiently proteolyze gluten by itself, so as to release peptide antigens of suitable length for T-cell presentation. In contrast, when rats were fed with whole-wheat bread coadministered with EP-B2 and SC PEP, the gluten immunotoxicity underwent a genuine reduction. These findings verify that EP-B2 and SC PEP can complement each other to digest gluten in whole-wheat bread before it is emptied into the small intestine (ie, the affected organ in patients with celiac sprue).

Discussion

Celiac sprue, or celiac disease, is an inheritable autoimmune disease of the small intestine that affects children and adults with high prevalence (1:100–1:300). If left untreated, it is associated with a variety of complications, including anemia, infertility, bone disorders, and intestinal lymphoma. The only accepted treatment for celiac disease is a strict, lifelong gluten-free diet. However, compliance to a strict gluten-free diet is difficult because of the ubiquitous nature of this protein, cross-contamination of foods, inadequate food labeling regulations, and social constraints. Nondietary therapies would be of considerable benefit to patients with celiac disease because the therapies could potentially allow these patients to safely incorporate modest amounts of gluten in their daily diet.

Glutenases are a promising class of therapeutic agents for celiac sprue. Early proof-of-concept experiments focused on the use of PEPs for gluten detoxification in the duodenum. PEPs were selected because of their ready commercial availability, although it was recognized that clinical implementation of these agents would require advanced drug delivery methods to promptly release high enzyme doses in the duodenum. More recent efforts have led to the identification of gastric glutenases that do not require sophisticated formulation and can detoxify dietary gluten before its release in the affected organ. At least 2 such purified glutenases, EP-B2 and a Aspergillus niger PEP, have been tested in the laboratory, and there is considerable evidence for the existence of other promising gastrically active glutenases in germinating cereals.

The promise of a combination glutenase that comprises a glutamine-specific endopeptidase and a proline-specific endopeptidase was recently highlighted using EP-B2 (under gastric conditions) and Flavobacterium meningosepticum PEP (under duodenal conditions). As discussed previously, both classes of enzymes are bona fide glutenases in their own right, because they have the ability to accelerate gluten digestion in the mammalian gastrointestinal tract. The key benefit of a combination product is that the enzymes can exhibit synergistic activity as a result of their complementary substrate specificity.

Here, we have presented the properties of a promising clinical candidate for treating patients with celiac disease—a combination glutenase that comprises EP-B2 and SC PEP. A practical advantage of this combination product is that both enzymes are active and stable in the stomach and can therefore be administered as lyophilized powders or simple capsules or tablets. In this combination, EP-B2 is primarily responsible for hydrolyzing complex gluten proteins at glutamine residues into relatively short (but still inflammatory) oligopeptides, whereas SC PEP breaks down these oligopeptides at internal proline residues to yield nontoxic metabolites. Assuming that an EP-B2 dose of 30 U/mg bread protein (corresponding to about 1:100 weight ratio of EP-B2/gluten) is clinically useful, our in vitro dose variation data suggests that a 1:1 weight ratio of EP-B2/SC PEP should maximize their synergistic potential, thereby greatly reducing the clinically effective dose of EP-B2. Initial animal trials showed vivo efficacy of the EP-B2 + SC PEP combination. Additional in vivo trials are required to establish the capacity of these enzymes to eliminate immunogenic peptides. Exploratory clinical studies on the actual benefit of such a combination glutenase therapy are clearly warranted in patients with celiac sprue.

Appendix

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1053/j.gastro.2007.05.028.

References


Received February 3, 2007. Accepted April 19, 2007.
Address requests for reprints to: Chaitan Khosla, PhD, 380 Roth Way, Keck Science Building, Stanford, California 94305, e-mail: khosla@stanford.edu; fax: (650) 723-6538.
Supported by a grant from the National Institutes of Health (DK 063158 to C.K.).
We thank Tina Riedel (Celiac Sprue Research Foundation, Palo Alto, CA) and Ryan Kelly (Mountain View, CA High School, Mountain View, CA) for their support in manufacturing the SC PEP enzyme. We also thank Harmit Vora (Stanford University), Jim McIntire (Alvine Pharmaceuticals), and Pawan Kumar (Alvine Pharmaceuticals) for supplying the EP-B2 enzyme for these experiments.
Chaitan Khosla is a founder and stockholder in Alvine Pharmaceuticals, which is developing the products described in this article for celiac sprue therapy. Andrew Spencer is a staff scientist and stockholder in Alvine Pharmaceuticals.