Effect of Barley Endoprotease EP-B2 on Gluten Digestion in the Intact Rat

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ABSTRACT

Celiac Sprue is a multifactorial disease characterized by an intestinal inflammatory response to ingested gluten. Proteolytically resistant gluten peptides from wheat, rye, and barley persist in the intestinal lumen and elicit an immune response in genetically susceptible individuals. Here, we demonstrate the in vivo ability of a gluten-digesting protease (“glutenase”) to accelerate the breakdown of a gluten-rich solid meal. The proenzyme form of endoprotease B, isoform 2 from *Hordeum vulgare* (EP-B2), was orally administered to adult rats with a solid meal containing 1 g of gluten. Gluten digestion in the stomach and small intestine was monitored as a function of enzyme dose and time by high-performance liquid chromatography and mass spectrometry. In the absence of supplementary EP-B2, gluten was solubilized and proteolyzed to a limited extent in the stomach and was hydrolyzed and assimilated mostly in the small intestine. In contrast, EP-B2 was remarkably effective at digesting gluten in the rat stomach in a dose- and time-dependent fashion. At a 1:25 EP-B2/gluten dose, the gastric concentration of the highly immunogenic 33-mer gliadin peptide was reduced by more than 50-fold within 90 min with no overt signs of toxicity. Evaluation of EP-B2 as an adjunct to diet control is therefore warranted in celiac patients.

Celiac Sprue, or celiac disease, is an inheritable, lifelong disorder of the small intestine that affects children and adults with high prevalence (1:100–1:300) (Pasano and Catassi, 2001; Maki et al., 2003). This multifactorial disease is characterized by an inflammatory response to ingested wheat gluten and similar rye and barley proteins, leading to damage of the intestinal villi and greatly reduced ability to absorb nutrients (Farrell and Kelly, 2002; Solid, 2002; Green and Jabri, 2003). Proline- and glutamine-rich gluten peptides from wheat, rye, and barley are relatively resistant to gastrointestinal digestion and persist in the intestinal lumen, allowing them to elicit an immunotoxic response in genetically susceptible individuals (Solid, 2002).

With approximately 15% proline and 35% glutamine residues, gluten is a unique dietary protein (Stern et al., 2001). Several studies have highlighted the therapeutic potential of prolyl endopeptidases (PEPs), an enzyme family that cleaves at internal proline residues, in detoxifying proteolytically resistant gluten epitopes (Hausch et al., 2002; Shan et al., 2002, 2004; Piper et al., 2004; Gass et al., 2005; Marti et al., 2005; Pyle et al., 2005). More recently, a complementary approach of using a glutamine-specific endoprotease (EP-B2, a cysteine endoprotease from germinating barley seeds) in conjunction with the PEP enzyme has demonstrated rapid detoxification of gluten under simulated gastrointestinal conditions (Bethune et al., 2006; Siegel et al., 2006). At a gluten/EP-B2/PEP weight ratio of 75:3:1, grocery store gluten was fully detoxified within 10 min of simulated duodenal conditions, as judged by chromatographic analysis, biopyl-derived T cell proliferation assays, and a commercial anti-gluten antibody test (Siegel et al., 2006).

The ultimate goal of this research is to demonstrate the ability of oral enzyme therapy to counter the toxic effects of moderate quantities of gluten ingested by Celiac Sprue patients. To facilitate evaluation of enzyme compositions, formulations, and dosing regimens, we sought to develop a rodent model for gluten digestion. A number of studies (Robert et al., 1991; Matsuda et al., 1993; Burton-Freeman et al., 1997; Izbeki et al., 2001; Overhaus et al., 2004; Turan and Ozdemir, 2004) have exploited the intrinsic similarities between gastrointestinal metabolism of food in rats and hu-
mans. Rats may be an effective model for gluten digestion because they are able to eat and digest a significant level of food (on the order of several grams, which would represent a physiological gluten load for a Celiac patient) in a suitable time frame (i.e., in approximately 5–8 h) (Kaneko et al., 1995).

The goals of this study were 2-fold: 1) to establish a model for gastrointestinal transit and digestion of gluten administered in solid form in adult rats and 2) to quantify the influence of oral enzyme supplementation on gluten breakdown in this model. Both of these goals were accomplished. Our findings provide a powerful new tool for pharmacological investigations into dietary gluten metabolism in mammals. They also vividly highlight our earlier proposals that gluten is a difficult protein to digest and that orally administered gluternases can greatly accelerate its breakdown and assimilation.

Materials and Methods

Materials. All materials used in the animal studies were food-grade or higher. Gluten was from Bob’s Red Mill (Milwaukee, OR), autoclavable rat chow was from Simonsen Labs (Gilroy, CA), white and brown sugar was from C&H Sugar Company (Crockett, CA), American cheese was manufactured by Kraft (Northfield, IL), sodium croscarmellose was from FMC (Philadelphia, PA), and vancomycin was from Sigma (St. Louis, MO). Trypsin (from bovine pancreas; T4665) and α-chymotrypsin (type H from bovine pancreas; C4129) were from Sigma. Using standard chromogenic assays, the specific activities of trypsin and chymotrypsin were 9000 and 2150 USP/mg, respectively. Substrates for the chromogenic assays were from Sigma, whereas the EP-B2 chromogenic substrate (Z-Phe-Arg-pNA) was from Bachem (Torrance, CA).

EP-B2 Enzyme Manufacturing and Testing. EP-B2 was prepared in a 10-liter fed-batch fermentation process that used a recombinant strain of Escherichia coli. The enzyme was purified from inclusion bodies via denaturing affinity chromatography, refolded, and concentrated in a similar manner as before (Bethune et al., 2006). Details of the scaled-up purification, refolding process will be described elsewhere (H. Vora and C. Khosla, manuscript in preparation). The final enzyme concentration was 14 to 18 mg/ml in 100 mM Tris-Cl, pH 8.0, 5 mM EDTA, 2 mM β-mercaptoethanol, and 15% sucrose. EP-B2 specific activity ranged between 1000 to 1500 U/mg.

Enzyme Activity Assays. All enzyme activity assays were performed in a similar manner as described earlier (Marti et al., 2005; Bethune et al., 2006). The specific activities of all enzymes were in the expected ranges based on previous literature.

Gluten Digestion in Rats. Adult male Wistar rats, weighing 260 to 280 g, were purchased from Simonsen Labs. Animals were acclimatized for a minimum of 2 days at the animal facility. Throughout the study, they were individually caged. All in vivo procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

The feeding regimen was developed to ensure rapid and reproducible ingestion of the solid gluten meal by the animals. During the 2-day acclimatization period, the animals were fed a standard diet of autoclavable rat chow and water. Two days before the study execution, rat chow was removed, and the animals were fed the solid gluten test meal to familiarize them to the gluten food source. The test meal was prepared by dissolving one part white sugar and one part brown sugar in one part water. Vancomycin (1 mg per 280 mg of gluten) was added as a nonabsorbable marker because its peptidic structure allows it to coelute with partially proteolyzed gluten peptides on an analytical HPLC column. Two parts gluten were then mixed into the sugar solution with a whisk to create a paste-like material. This dough was then baked in an oven for approximately 15 to 20 min at 300–350°C to yield a product with a cookie-like consistency. The animals were fasted overnight (approximately 8–14 h) on the penultimate day before the trial. After this fast, the animals were fed the gluten/sugar cookie for approximately 8 to 12 h. This step was performed to train the animals to eat the gluten/sugar cookie immediately after a fast. The animals were then fasted overnight (minimum of 12 h; maximum of 24 h) before the trial execution. For this fast, the animals were placed on wire bottom cages to minimize coprophagia.

On the day of the trial execution, the animals were administered the gluten-sugar test meal. In most cases, they consumed 2 to 6 g of meal within 15 to 45 min. It is noteworthy that with the exception of two cheese-fed control animals, gluten was the only source of protein in the test meal; hence, the luminal content of the rats contained only gluten-derived peptides. At specified periods after the start of feeding (90, 150, or 210 min), the animals were sacrificed by carbon dioxide gas, followed by cervical dislocation. The gastric and small intestinal contents were collected. Gastric samples were scraped from the dissected stomach, placed in a 15-ml Falcon tube (VWR, West Chester, PA), and frozen immediately in a dry ice/ethanol bath. Small intestinal samples were collected using saline solution (~1 ml) to individually flush out the contents of the duodenal (defined as the first 10 cm of the rat intestine), jejunal, and ileal segments. The jejunal and ileal segments were obtained by splitting the remainder of the small intestine (approximately 80 cm) into two equal pieces, defining the proximal section as the jejunum and the distal section as the ileum. Whereas the contents of the stomach had the consistency of a soft, fibrous paste, the small intestinal contents had a more fluid-like character. All intestinal samples were collected in 15-ml Falcon tubes, quick-frozen in a dry ice/ethanol bath, and stored at ~80°C before analysis.

At the time of analysis, all of the frozen intestinal samples were thawed in a <10°C water bath and then centrifuged at 2800g and 4°C for 10 min. The gastric samples were thawed and resuspended at 0.01 N HCl before centrifuging. A 200-μl aliquot was taken from each sample, and the aliquot was heat-deactivated at 95°C for >5 min. The aliquot was then centrifuged at approximately 14,000g for 10 min and syringe-filtered using a 0.2-μm filter, and the supernatant was analyzed by reverse phase HPLC as described below. In addition, trypsin and chymotrypsin enzyme activities were determined on initially thawed intestinal samples for select conditions, and their activity was compared against appropriate controls (i.e., the activity of the purified enzyme purchased from Sigma). All trials were performed at least triplelicate, except for the cheese-fed animals, which were studied in duplicate.

The distribution of the ingested meal at the time of euthanization in the gastrointestinal tract was estimated for each animal. For the stomach material, the wet weight of each sample was measured. For the intestinal samples, the area under the curve for the vancomycin (nonabsorbable intestinal marker) was determined. This area under the curve was converted to a “% meal retained” by using the vancomycin calibration curve, a nominal overall sample volume of 1.5 ml, and the incoming level of vancomycin in the food (1:280 vancomycin/gluten weight ratio).

Effect of EP-B2 on Gluten Digestion in Rats. Three doses of EP-B2 [1:25, 1:50, and 1:100 EP-B2/gluten (w/w)] were investigated to determine the dose dependence of gluten digestion by EP-B2. For each dose, a 1-g gluten meal was fed to the rats, followed by either a 30- or 90-min digestion period. The 30-min time point was selected to evaluate the ability of EP-B2 to activate and digest gluten at the start of the digestive process, because EP-B2 is administered as a proenzyme that is activated under acidic conditions. It should be noted that not all of the animals completely consumed their 5-g meal within the given time limit (four of eight unfinished at 30 min; one of eight unfinished at 90 min), but all of the animals ate at least approximately half of their meal. The 90-min time point was used to study EP-B2 efficacy because the enzyme is expected to be fully
active at this time. One of the 90-min animals [at a 1:25 EP-B2/gluten (w/w)] was removed from the study because this animal was observed to eat its meal in two distinct portions, with the second portion (~1/3 of the meal) eaten between 60 to 75 min. As such, the digestive products for this animal were not representative of an extended digestive time. All other 90-min animals did not eat a significant portion of their meals after the 60-min time point. At the highest EP-B2/gluten dose (1:25), an intermediate digestion time of 60 min was also evaluated.

To evaluate EP-B2 efficacy in rats, the protocol described above was followed with a few modifications. In all cases, the animals were challenged with 1 g of gluten because this lower gluten minimized enzyme requirements. For facile administration of the desired EP-B2 dose, the composition of the meal was slightly modified to absorb additional liquid associated with the enzyme formulation. The final meal composition (by weight) was 12% brown sugar, 12% white sugar, 49% EP-B2 solution, 20% gluten, and 7% croscarmellose sodium (Ac-Di-Sol). The croscarmellose sodium was added to increase the absorptive capacity of the mixture. The concentration of the EP-B2 solution was adjusted to target the appropriate dose. Each animal was fed 5 g of the gluten-sugar test meal, administered uncooked to avoid enzyme denaturation. In addition, female Sprague-Dawley rats (~300 g) were used in these trials. (The use of a different rat breed was required because of a change in animal facility and did not affect the validity of the experiments.) Last, the testing of freshly thawed gastric samples was modified slightly to ensure that no EP-B2 digestion occurred ex vivo. Gastric material testing of freshly thawed gastric samples was modified slightly to avoid enzyme denaturation. In addition, female animal was fed 5 g of the gluten-sugar test meal, administered 1 h before sampling. Samples (200 μl) were handled as detailed above. All other experimental conditions (e.g., fasting protocols, sampling procedures, and HPLC analysis) were performed in an identical fashion as above.

**Reverse-Phase HPLC.** Samples harvested from the stomachs or small intestines of rats were chromatographically separated on a 4.6 × 150-mm reverse-phase C18 protein and peptide column (Grace Vydac, Hesperia, CA) using a Varian-Rainin Dynamax (Palo Alto, CA) SD-200 pumps (1 ml/min), a Varian 340 UV detector set at 215 nm, and a Varian Prostar 430 autosampler. Solvent A was water with 0.01 N HCl and 50 μl of 10 mM leupeptin (a known inhibitor of EP-B2). This material was incubated at 37°C, pH 2.5, for 10 min to allow the gluten content to dissolve. After 10 min, pepsin (0.6 mg/ml final concentration) was added to the gluten suspension; this step was found to be necessary to ensure that all the gluten content of the stomach was chromatographically analyzed. The mixture was further incubated at 37°C for an additional 30 min before sampling. Samples (200 μl) were handled as detailed above. All other experimental conditions (e.g., fasting protocols, sampling procedures, and HPLC analysis) were performed in an identical fashion as above.

**Mass Spectrometry Assay for Representative Immunotoxic Gluten Oligopeptide (33-mer).** The level of a proline and glutamine rich 33-mer peptide from α-gliadin, LQLQPPQPQPQLYP-PQQLPYQPQPQLYPQPQP, in gastric samples was determined by a mass spectrometry assay. The 33-mer peptide was selected for analysis because of its extreme resistance to breakdown by luminal proteases and its potent ability to trigger T cell proliferation from small intestinal biopsies from DQ2 Celiac Sprue patients (Shan et al., 2002; Qiao et al., 2004). Gastric material (100 mg) was re-suspended with a spatula in 950 μl of 0.01 N HCl and 50 μl of 10 mM leupeptin (a known inhibitor of EP-B2). This material was incubated at 37°C, pH 2.5, for 10 min to allow the gluten content to dissolve. After 10 min, 100 μl of 500 mM Na2HPO4 buffer, pH 6, and 35 μl of 1 M NaOH was added to the mixture to increase the pH to >6. Pancreatic enzymes, trypsin, and chymotrypsin (0.375 mg/ml final concentration) were then added to the gluten suspension. This step was required because the 33-mer is a product of pancreatic digestion. The mixture was incubated at 37°C for an additional 30 min, and the mixture was then heated at 95°C for >5 min to deactivate the enzymes. The material was centrifuged for 10 min at approximately 14,000g. An aliquot was analyzed by HPLC as described previously to determine its vancomycin quantity. Before mass spectrometry analysis, all samples were precipitated with acetonitrile. A 50-μl aliquot from each sample was mixed with an equal volume of acetonitrile, vortexed, refrigerated at 4°C for at least 2 h, and centrifuged at 14,000g for 10 min at 4°C.

**LC-MS/MS quantitation studies were performed on the supernatant.** Duplicate injections were performed for each sample. Gastric samples from each animal were evaluated, and duplicate samples for all 30-min digestion time rats were evaluated. An Agilent 1100 HPLC system (Agilent Technologies, Inc., Palo Alto, CA) was used for solvent delivery and sample introduction. Samples (10 μl) were injected onto a reverse-phase column (Luna C18 Phenomenex, Torrance, CA). Solvent A was water with 0.1% formic acid (v/v). Solvent B was acetonitrile with 0.1% formic acid (v/v). The LC eluate was directed to waste for the first 1.5 min of each analytical run and thereafter was introduced into the mass spectrometer ion source without splitting. A Waters Micromass Quattro Premier triple quadrupole mass spectrometer (Waters, Milford, MA) equipped with a standard electrospray ion source was used for detection. The mass spectrometer was operated with a capillary voltage of 3.5 kV, source temperature of 120°C, desolvation temperature of 350°C, and collision gas pressure of 4.2 × 10⁻³ mbar. Positive ion selected reaction monitor mode was used for monitoring the transitions of ions at m/z 978.94→263.4⁺ (30 V cone voltage, 27 eV collision energy) for the quantitation assay and m/z 1304.71→263.4⁺ (40 V cone voltage, 50 eV collision energy) as a confirmatory transition. The limit of quantitation for the 33-mer was found to be 10 fmol/μl for jejunal samples. Standard calibration curves were generated using synthetic 33-mer peptide at concentrations ranging from 0.03 to 2 pmol/μl. Instrumentation control and data analyses were accomplished using MassLynx 4.0 and QuanLynx 4.0 software (Waters). All results were normalized by the vancomycin levels in the sample. LC-MS/MS analysis of jejunal samples was also performed using the same analytical method.

**Results**

**Gluten Digestion in Rats.** Fasted rats were administered 2 or 4 g of a gluten-sugar test meal (50% gluten, by weight). Animals were allowed approximately 90, 150, or 210 min (high-dose only) to consume and digest the meal before euthanasia. The gastrointestinal contents were recovered, processed, and analyzed by HPLC as described under Materials and Methods.

**Gastrointestinal Distribution of Gluten.** The distribution of gluten within the gastrointestinal tract was determined by weighing the wet contents of the stomach and by measuring the amount of vancomycin recovered from the small intestinal samples. In most experiments, an appreciable portion of material remained in the stomach (Table 1). For the low-dose animals, >1 g of the wet material remained in the stomach at 90 min, whereas the majority of the material had emptied by 150 min. For the high-dose animals, minimal material had emptied the stomach at the 90-min time point, and approximately 50% had emptied at 150 and 210 min. The distribution of the material in the small intestine was determined by measuring the amount of vancomycin in the sample compared with the total in the ingested meal. Although duodenal contents contained only small amounts of the ingested meal, appreciable quantities of food were recovered from the jejunum and ileum. At the 90-min digestion time, comparable amounts of material were recovered from the lumen of the jejunum and ileum. At the 150- and 210-min digestion times, more material could be observed in the ileum (Table 1). Overall, 9 to 17% and 8 to 24%...
of the ingested vancomycin was recovered in the jejunum and ileum, respectively.

**HPLC Analysis of Gastrointestinal Samples.** Gastric and intestinal samples from each animal were analyzed by HPLC as detailed under Materials and Methods. Several general features of the HPLC traces should be noted (Marti et al., 2005; Gass et al., 2005). Due to the complex composition of whole gluten, most peaks correspond to multiple peptides. Generally speaking, shorter peptides elute earlier, whereas longer peptides have longer retention times. Some of these digestion products have been characterized using ion trap mass spectrometry (Marti et al., 2005). Based on in vitro gluten digestions, it is likely that peaks eluting before 10 min represent primarily end products (2-6-mers) of pancreatic protease digestion because the magnitudes of these peaks increase uniformly as a function of pancreatic digestion time. Peptides eluting after 15 min are likely to include most immunotoxic peptides in partially digested gluten, because representative peptides of 11 (PFPQPQLPYPQP), 14 (PQPQLPYPQPLPY), and 28 (PFPQPQLPYPQPLPYPQPLPY) residues elute at 18.5, 20, and 22 min, respectively (Gass et al., 2005). Soluble gluten (without enzymatic digestion) elutes at 25 min; this 25-min peak most likely represents a mixture of soluble gluten protein and large gluten-derived peptides (>30 residues). Last, a limitation of the HPLC analysis is that a full material balance for the gluten cannot be performed as only soluble gluten will be analyzed, and large soluble gluten proteins may be captured on the guard column and not represented on the HPLC trace.

In all cases, the HPLC profiles of gastric contents (Fig. 1) were similar to gluten digested with pepsin in vitro (Gass et al., 2005), with a large, late eluting peak at approximately 25 min (representing soluble gluten) and additional smaller peaks eluting at 18 to 24 min. For the low-dose animals (1 g of gluten; 2 g total), near-complete emptying of the stomach is observed at 150 min, as indicated by a reduction in both gluten and vancomycin peaks (approximately 8 min). For the high-dose animals (2 g of gluten; 4 g total), as expected, the overall magnitude of the gluten peaks is approximately double that of the low-dose animals.

The small intestinal contents of the gluten-dosed animals were evaluated in three intestinal regions: duodenal, jejunal, and ileal. Interestingly, no gluten was identified in the duodenal segments of any animal, regardless of gluten dose or time after administration. This is indicated by the absence of any significant peaks in the HPLC trace in the 12- to 22-min range, suggesting that transit of the test meal through the duodenum is rapid, regardless of the amount of food.

In contrast to the duodenal samples, a strong and reproducible gluten signal is observed in both the jejunal and ileal samples. The abundance and composition of partially proteolyzed gluten in rat jejunal samples is shown in Fig. 2. All jejunal results display a similar profile and magnitude, independent of the starting dose and the digestion time. The overall profile of the digested gluten is remarkably similar to that of in vitro digested gluten (Gass et al., 2005). In particular, a broad envelope is observed in the 12- to 22-min range. Earlier studies have shown that this envelope is comprised of a large number of 8- to 30-mer gluten peptides (Gass et al., 2005; Marti et al., 2005). One difference between the in vitro and in vivo gluten digestions is the presence of the sharp, late eluting peaks observed in the in vivo samples (Fig. 2; 22- to 26-min range). These peaks are most likely bile salts (Hayashi et al., 1985; Sakakura et al., 1993). This hypothesis is supported by the fact that these peaks can be removed by the addition of cholestyramine resin (a bile salt-sequestering agent) to the intestinal samples (data not shown). The bile

**Fig. 1.** Gastric contents of rats dosed with low-dose (1 g) gluten, 90 (light gray line) and 150 min (medium gray line) after administration of the test meal. Both the peak at 25 min and the smaller, broader peak centered at 22 min are characteristic signatures of pepsin-digested gluten (Gass et al., 2005). Vancomycin (V) is the nonabsorbable volume marker. N-Tosyl-L-arginine methyl ester (T) is the internal HPLC standard.
salt peaks increase appreciably in magnitude in the jejunum compared with the duodenum.

The ileal contents of rats dosed with low- (1 g) and high-dose (2 g) gluten were examined (data not shown). For the low-dose animals, the peak magnitude in the 12- to 22-min range is considerably lower, and the vancomycin peak is significantly more prominent in the ileum than that observed in the jejunum. These observations indicate that low quantities of a solid meal are fully absorbed as it passes down the intestine before reaching the ileum. For the high-dose animals, gluten peptides eluting in the 12- to 22-min range are observed, particularly at the 150 and 210 min after ingestion. At the high-dose conditions, the digestive capacity of the upper part of the small intestine is exceeded, allowing partially digested material to reach the ileum. The abundance of gluten oligopeptides varies as a function of digestion time. At 90 min, gluten oligopeptides are present at relatively low levels in the ileum, indicating that the excess gluten has not yet reached the distal small intestine. The abundance of gluten oligopeptides peaks at 150 min and is then reduced at the 210 min, and the magnitude of the vancomycin marker increases slightly from 150 to 210 min, reflecting concentration due to solute and water absorption. This suggests that the gluten is primarily processed at more proximal levels of jejunum and upper ileum.

**Non-Gluten Control Animals.** Animals fed a gluten-free meal containing a comparable amount of protein (4 g of cheese) were evaluated to verify that the proteolysis profile described above is unique to gluten. As expected, the resulting HPLC traces for the cheese-fed and gluten-fed animals were entirely different (Fig. 3). From the cheese-fed animals, additional early eluting peaks were observed in the gastric samples, suggesting that pepsin-catalyzed proteolysis of milk protein was fairly extensive (data not shown). The small intestinal samples for the cheese-fed animals lacked the characteristic gluten peptide region of the gluten-fed animals (Fig. 3). Indeed, baseline traces were observed in the 12- to 22-min region for the jejunum and ileal samples from cheese-fed animals, indicating extensive digestion of the milk proteins to smaller peptide products. It is noteworthy that vancomycin and bile salt associated peaks for the cheese-fed animals were of comparable magnitude to that of the gluten-fed animals.

**Animal-to-Animal Reproducibility.** The reproducibility of the above data were verified for all experimental conditions. To quantify animal-to-animal variability in the jejunal

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**Fig. 2.** Jejunal contents of rats dosed with low-dose (1 g; Fig. 3) gluten, 90 (light gray line) and 150 min (medium gray line) after administration of the test meal. The broad envelope in the 12- to 20-min range is a characteristic signature of gluten that has been subjected in vitro to pepsin followed by pancreatic protease digestion (Gass et al., 2005). The sharp peaks eluting after 20 min are most likely due to various bile salts in the intestinal lumen; their abundance is constant, regardless of gluten dose or time of sacrifice. Similar results are observed for the high-dose (2 g of gluten) animals (data not shown). Standard peaks are defined in legend to Fig. 1.

**Fig. 3.** Jejunal contents of rats fed with gluten (dark gray line) or cheese (light gray line) test meal. In both cases, the animals were fed approximately 4 g of the solid meal. The intestinal samples for the cheese-fed animals lack the characteristic gluten peptide region (diffuse peak; 18–23 min) of the gluten-fed animals. The jejunal HPLC trace for the cheese-fed animal is essentially flat in the 12- to 22-min region, indicating essentially complete processing of the cheese-associated proteins. It is noteworthy that vancomycin (V; 8.5 min) and bile salt associated peaks (after 20 min) for the cheese-fed animals are of comparable magnitude with that of the gluten-fed animals. Standard peaks are defined in legend to Fig. 1.
and ileal samples, the area under the curve from 12 min until the start of the bile-associated peaks (~20–22 min) (representative of the gluten oligopeptide region) was calculated and normalized by the vancomycin peak (eluting at approximately 7–8 min). Overall, digestion of gluten in the test meal was reproducible for all of the experimental conditions (% relative standard deviation ranging from 7–21%), with the exception of the jejunal samples for the low-dose 150-min animals (% relative standard deviation of 49%). This high variability may be a result of the near-complete emptying of the stomach in these animals.

Pancreatic Enzyme Analysis. In addition to HPLC analysis of the above samples, pancreatic enzyme activities were measured from the intestinal flush samples for the gluten-fed animals. Chymotrypsin and trypsin activities were measured in intestinal flush samples from at least one rat in each cohort. For all samples, enzyme activity was significantly less in the duodenum than in the jejunum and ileum, both of which had comparable activity levels (Table 2). This observation matched well with earlier literature reports (Pelot and Grossman, 1962). No correlation was observed between the experimental conditions (gluten load or digestion time) and the level of pancreatic enzymes (Table 2).

**TABLE 2**

Pancreatic enzyme levels in small intestinal samples of gluten-fed rats

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<th>Low Dose</th>
<th>High Dose</th>
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<td></td>
<td>90 Min 150 Min</td>
<td>90 Min 150 Min 210 Min</td>
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<tr>
<td>Chymotrypsin (mg/ml)</td>
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**Fig. 4.** Gastric contents of rats fed with gluten (1 g) test meal with varying levels of EP-B2 enzyme, followed by 90-min digestion time: control (no EP-B2) (dark gray line), low-dose EP-B2 (10 mg) (medium gray line), and high-dose EP-B2 (40 mg) (light gray line). The HPLC traces are divided into early peaks (representing smaller peptide products), middle peaks, and late peaks (representing larger undigested gluten-derived peptides and soluble gluten protein). The approximate elution time of the 33-mer peptide is 25 min. Standard peaks are defined in legend to Fig. 1.
The level of 33-mer peptide in each of the gastric samples was determined by mass spectrometry (Fig. 5, A and B). The results show a decrease in the 33-mer peptide concentration at the 90-min digestion for all three EP-B2 doses. A 1:25 EP-B2/gluten (w/w) dose resulted in a greater than 50-fold reduction in the 33-mer peptide levels compared with untreated controls. At 30-min digestion times, there was no significant difference observed in the 33-mer peptide levels for any of the EP-B2 doses versus that of the control samples. The dramatic reduction in 33-mer peptide levels at 90 min is even greater than that reflected by the area-under-the-curve values of the 23.5- to 26-min peaks, which contains the 33-mer peptide (vide supra). One potential reason for this difference is the specificity of EP-B2 toward sequences found in the 33-mer peptide as well as many other immunotoxic gluten peptides. EP-B2 has considerable specificity for the sequence Q↓XP, which is abundant in the 33-mer as well as many other immunotoxic gluten sequences (Bethune et al., 2006). In addition, we have observed that EP-B2 substantially solubilizes gluten in the gastric samples because the gastric samples from the EP-B2 animals had a more liquid-like consistency than the controls. By facilitating the gluten solubilization, it is likely that more gluten has been dissolved and captured by the HPLC analysis for the EP-B2 animals than for the control animals. This would result in a higher relative area for the 23.5- to 26-min peak for the EP-B2 animals than for the controls, where minimally proteolyzed gluten would be retained on the guard column.

The amount of material that emptied from the stomach at both the 30- and 90-min time points was determined by summing the vancomycin levels of the jejunal and ileal samples. On average, approximately 9 and 21% of the initial vancomycin (and hence, gluten load) was recovered from the intestine for the 30- and 90-min digestion times, respectively.

In addition, samples from the jejunum from all of the EP-B2 experimental animals were analyzed for 33-mer levels. A significant portion of the samples (22%; four of 18) had values below the limit of detection. For all of the animals, the 33-mer levels, when normalized for vancomycin amounts, were several orders of magnitude lower than that of the stomach samples (ranging from 0.002–0.1% of the stomach sample levels). No trends with respect to the EP-B2 dosage were observed.

**Time Dependence of High-Dose EP-B2 [1:25 EP-B2/Gluten (w/w)].** A comparison of three digestion times (30, 60, and 90 min) was performed at the high EP-B2 dose. The digestion times represented a maximum time for digestion because the animals require a minimum of 10 to 15 min to consume the gluten meal. HPLC results revealed that the extent of digestion by EP-B2 is proportional to the digestion time. This was shown by both the rate of product formation (4- to 9-min peaks) and soluble gluten depletion (25-min peak) (data not shown). The level of 33-mer peptide also decreased as the digestion time was increased, showing a greater than 5-fold decrease at 60 min and a greater than 50-fold decrease at 90 min (Fig. 5C).

**Discussion**

An understanding of gluten digestion in an appropriate animal model is critical to the development of an efficacious oral enzyme therapeutic agent for Celiac Sprue. Earlier studies have mimicked human gastric and small intestinal conditions ex vivo or have used jejunal intubation protocols to investigate glutenase activity (Haasch et al., 2002; Shan et al., 2002; Piper et al., 2004; Gass et al., 2005; Marti et al., 2005; Matysiak-Budnik et al., 2005; Pyle et al., 2005; Bethune et al., 2006; Siegel et al., 2006). Here, we have evaluated in vivo the ability of a target enzyme to digest gluten that has been ingested as part of a solid meal. Our results have important implications for the pharmacokinetic and pharmacodynamic behavior of EP-B2 in Celiac Sprue patients.

One of the primary goals of this study was to establish a model for gastrointestinal transit and digestion of gluten administered in solid form in adult rats. This was established by first developing a reproducible and straightforward animal handling and feeding protocol. Adult rats (260–280 g) were received into the animal facility, acclimated to eating a gluten-rich diet (50% by weight; sole protein source), and evaluated for their digestion of a fixed gluten-rich meal, all within 5 days upon receiving the animal. One reason for

Fig. 5. 33-mer peptide levels of gastric contents of rats fed with gluten (1 g)/sugar solid meal for various digestion times with varying levels of EP-B2 enzyme. Samples from all animals were tested in at least duplicate. The error bars represent 1 S.D. for all test results. All of the results were normalized by the area of the nonabsorbable marker (vancomycin), and the number of rats evaluated is presented above the respective bar. a, 33-mer peptide levels of gastric contents of rats fed with gluten (1 g)/sugar solid meal and high-dose EP-B2 animals [1:25 EP-B2/gluten (g/g)]. Each bar corresponds to a different duration of digestion (30–90 min).
selecting adult rats for this experiment was their ability to ingest large quantities of gluten (1–2 g) in a relatively short time frame (1–4 h). This high level of gluten is advantageous because this gluten load is similar to the gluten load (5 g) that has been shown to induce malabsorption in celiac patients in a short time period (i.e., 2 weeks) (Pyle et al., 2005).

The extent of gluten proteolysis was determined in the stomach and small intestine for a variety of gluten loads (1–2 g) and digestion times (90–210 min). In all cases, the HPLC profiles of gastric (Fig. 1) and intestinal (Fig. 2) contents were similar to gluten digested in vitro with the appropriate gastric and pancreatic enzymes (Gass et al., 2005). A combination of low pH and pepsin facilitates limited solubilization and proteolysis of gluten in the stomach as indicated by the large, late eluting peak at approximately 25 min, which has been attributed to initial soluble gluten and large gluten-derived peptides (Fig. 1). Thereafter, most of the subsequent breakdown of gluten occurs in the middle and lower small intestine. The transit time of partially digested food in the rat duodenum is very short (<30 min), independent of the amount of food ingested. This was demonstrated by the absence of any significant peaks in the HPLC trace in the 12- to 22-min range (indicative of gluten oligopeptides) or at 25 min (indicative of initial soluble gluten) for all experimental conditions (data not shown).

The HPLC results for the jejunal samples all display a broad envelope of peaks in the 12- to 22-min range of a similar magnitude, independent of the starting dose and the digestion time (Fig. 2). This profile is similar to that of gluten digested in vitro sequentially with pepsin and then with pancreatic proteases, trypsin, chymotrypsin, elastase, and carboxypeptidase A (Gass et al., 2005). Earlier studies have shown that this envelope is comprised of a large number of 8- to 30-mer gluten oligopeptides (Gass et al., 2005; Marti et al., 2005). This suggests that although food traverses the duodenum rapidly, it is well mixed with pancreatic protease secretions and that their action on gluten is rapid. The sharp peaks observed in the 4- to 8-min range were also observed in vitro when using pepsin, followed by trypsin, chymotrypsin, elastase, and carboxypeptidase A (Gass et al., 2005). These peaks most likely correspond to dipeptides and triptides released via the action of the pancreatic proteases in concert with terminal trimming by the intestinal brush-border surface membrane peptidases.

For the ileum samples, minimal gluten-associated peaks are observed at the 1 g of gluten dose, indicating that low doses (<1 g) of gluten are mostly digested and assimilated by the time the test meal arrives in the ileum. The relative importance of ileal digestive processes increases with increasing gluten dose. Up to 1 g of gluten can be mostly absorbed by the ileum within 1 h, although higher gluten doses (>2 g) may require more than 3 h (data not shown).

The above results were confirmed to be unique to gluten by evaluating the digestion of nongluten dietary proteins (milk protein) in the animal model. Digestion of nongluten dietary protein is much faster than gluten digestion. For example, the breakdown of milk protein is more extensive in the stomach (data not shown), as indicated by the presence of significant early eluting peaks, and it is efficiently digested by the time it arrives in the jejunum (Fig. 3), as shown by the absence of any late eluting peaks (>10 min). Our findings provide a new tool for pharmacological investigations into dietary gluten metabolism in mammals and reinforce our earlier findings that gluten is a difficult protein to digest.

The rat is an excellent model for the gastric phase of protein digestion, and it presents an opportunity to evaluate glutenases, such as barley cysteine endoprotease EP-B2, that are targeted to the stomach. The efficacy of EP-B2 was evaluated at a range of doses and digestion times. Recent in vitro studies have demonstrated that barley endoprotease EP-B2 is able to digest gluten under gastric conditions (Bethune et al., 2006; Siegel et al., 2006). This is advantageous because Celiac Sprue affects the upper small intestine, and thus, the gluten toxicity can be eliminated before the site of disease pathogenesis. Here, we have shown that EP-B2 facilitates digestion of the solid gluten-rich meal in vivo at doses as low as 1:100 EP-B2/gluten (w/w) and at time points as short as 60 min. A drastic reduction in the levels of the dominant, immunotoxic 33-mer peptide occurred at a 1:25 EP-B2/gluten (w/w) dose at both 60 min (>5-fold reduction) and 90 min (>50-fold reduction) (Fig. 5). This demonstrates that EP-B2 can detoxify the vast majority of gluten that is present in the meal, because 75 to 80% of the solid meal could be recovered from the stomach after 90 min.

One concern with targeting gluten digestion and detoxification in the stomach with a self-activating proenzyme is ensuring that the material that is initially released from the stomach has been properly digested. These animal studies have shown that minimal material gets released from the stomach before 30 min (<10% of the overall meal, based on intestinal vancomycin levels), and 75 to 80% of the meal remains in the stomach at 90 min, at which time the enzyme has been sufficiently activated to reduce the amount of toxic gluten oligopeptides (Table 1). As such, the vast majority of the gluten in the ingested meal is exposed to activated enzyme and can be fully detoxified. An alternative approach may involve using a coformulation of EP-B2 with a complementary enzyme, such as a gastric stable prolyl endopeptidase (Edens et al., 2005; Lopez and Edens, 2005). Overall, our results demonstrate the in vivo performance of a therapeutically promising enzyme for the detoxification of gluten.

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