Randomised clinical study: *Aspergillus niger*-derived enzyme digests gluten in the stomach of healthy volunteers

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SUMMARY

Background
Aspergillus niger prolyl endoprotease (AN-PEP) efficiently degrades gluten molecules into non-immunogenic peptides *in vitro*.

Aim
To assess the efficacy of AN-PEP on gluten degradation in a low and high calorie meal in healthy subjects.

Methods
In this randomised, double-blind, placebo-controlled, cross-over study 12 healthy volunteers attended to four test days. A liquid low or high calorie meal (4 g gluten) with AN-PEP or placebo was administered into the stomach. Via a triple-lumen catheter gastric and duodenal aspirates were sampled, and polyethylene glycol (PEG)-3350 was continuously infused. Acetaminophen in the meals tracked gastric emptying time. Gastric and duodenal samples were used to calculate 240-min area under the curve ($AUC_{0-240\ min}$) of α-gliadin concentrations. Absolute α-gliadin $AUC_{0-240\ min}$ was calculated using duodenal PEG-3350 concentrations.

Results
AN-PEP lowered α-gliadin concentration $AUC_{0-240\ min}$, compared to placebo, from low and high calorie meals in stomach (low: 35 vs. 389 µg × min/mL; high: 53 vs. 386 µg × min/mL; $P < 0.001$) and duodenum (low: 7 vs. 168 µg × min/mL; high: 4 vs. 32 µg × min/mL; $P < 0.001$) and absolute α-gliadin $AUC_{0-240\ min}$ in the duodenum from low (2813 vs. 31 952 µg × min; $P < 0.001$) and high (2553 vs. 13 095 µg × min; $P = 0.013$) calorie meals. In the placebo group, the high compared to low calorie meal slowed gastric emptying and lowered the duodenal α-gliadin concentration $AUC_{0-240\ min}$ (32 vs. 168 µg × min/mL; $P = 0.001$).

Conclusions
AN-PEP significantly enhanced gluten digestion in the stomach of healthy volunteers. Increasing caloric density prolonged gastric residence time of the meal. Since AN-PEP already degraded most gluten from low calorie meals, no incremental effect was observed by increasing meal caloric density. Clinical-Trials.gov, Number: NCT01335503; www.trialregister.nl, Number: NTR2780.

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INTRODUCTION

Gluten is a storage protein present in wheat, barley and rye and is exceptionally rich in proline, rendering gluten peptides resistant to gastrointestinal digestion. Proline-cleaving proteases are absent in the human gastrointestinal tract. Therefore, long proline-rich gluten peptides reach the small intestine intact after ingestion.[1] About 1% of the western population is suffering from coeliac disease.[2–5] In these patients, exposure of duodenum and proximal small intestine to the specific amino acid sequences of such poorly digested proline-rich gluten peptides triggers an abnormal immune response. This causes inflammation with infiltration of lymphocytes in the intestinal mucosa and ultimately villous atrophy and crypt hyperplasia.[6] Adverse reactions to gluten consumption are not limited to subjects suffering from coeliac disease. Presently non-coeliac gluten sensitivity has been clinically recognised as a separate condition in which neither allergic nor autoimmune mechanisms are involved.[7, 8] The symptoms experienced by these subjects are often identical to those seen in coeliac disease.[9, 10]

A lifelong gluten-free diet is the only treatment for individuals who cannot tolerate gluten. However, a gluten-free diet is hard to comply with as gluten-free products may not always be labelled, and may not always be at hand during social events or travelling.[11–13]

Prolyl endopeptidases belong to a family of enzymes with the ability to cleave at internal proline residues within a peptide.[14] Early investigations on oral protease therapy as an approach to degrade gluten have focused on bacterial prolyl oligopeptidases.[15–18] However, several *in vitro* studies conducted with such enzymes revealed only low enzymatic activity at acidic stomach pH and rapid degradation of these enzymes by pepsin.[16] Moreover, these enzymes were not able to prevent passage of potentially harmful gluten fragments into the small intestine.[16, 19] But, other enzymatic preparations have shown to be capable of degrading complex gluten proteins both *in vitro* and *in vivo*.[20–23]

In this respect, the *Aspergillus niger*-derived prolyl endoprotease (AN-PEP) also presents a promising option to degrade inadvertent dietary gluten. The use of the enzyme as food supplement has undergone successful evaluation by the French Agency for Food, Environmental and Occupational Health & Safety.[24] AN-PEP is active between pH 2 and 6, with optimum activity at gastric pH between 3 and 5.[19] In a dynamic, multi-compartmental gastrointestinal *in vitro* model, AN-PEP was shown to degrade almost all immunogenic gluten epitopes from gluten-containing meals into non-immuno-genic fragments during passage through the stomach compartment.[25] A pilot study in coeliac patients showed that a combination of AN-PEP and gluten was safe and well tolerated.[26] Before AN-PEP can be used as a future digestive aid for subjects intolerant to gluten, it is essential that the promising *in vitro* results are confirmed in a human *in vivo* study, focussing on duodenal gluten delivery after intake of gluten-containing meals with and without AN-PEP. Thereafter, the efficacy and safety of AN-PEP should be evaluated in target populations.

Our aim was to investigate the efficacy of AN-PEP on gluten degradation in an intragastrically delivered gluten-containing meal in healthy volunteers. To standardise meal intake, we administrated the meal intragastrically at a fixed rate instead of by oral ingestion. Secondly, we hypothesised that increasing the caloric density of a meal enhances gluten degradation by delaying gastric emptying and thereby prolonging gastric residence time of the meal.

MATERIALS AND METHODS

The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center (MUMC+) and conducted in full accordance with the principles of the Declaration of Helsinki of 1975 as revised in 2008 and with the Dutch Regulations on Medical Research involving Human Subjects (WMO, 1998). The study was performed at the MUMC+ from December 2011 to May 2012. All participants gave their written informed consent before participation. The trial has been registered in the Clinical Trials register (NCT01335503) and the Dutch trial register (NTR2780).

SUBJECTS

Healthy volunteers aged 18–45 years were recruited by advertisement. All subjects were screened by means of a standardised general physical examination. Reasons for exclusion included: history of gastrointestinal disorders or gastrointestinal surgery interfering with gastrointestinal function; history of major disease, use of medication (except oral contraceptives) within 14 days before testing; dieting; pregnancy; lactation; excessive alcohol consumption (>20 alcoholic consumptions/week) and smoking.

Design and intervention

In this double-blind, randomised, placebo-controlled, crossover study, participants attended to four test days with at least 1 week washout period between two test days. At test days, participants were randomised in a

double-blind fashion to 1 of the 24 possible orders of the four interventions; a low calorie gluten meal with AN-PEP or placebo, or a high calorie gluten meal with AN-PEP or placebo. The randomisation list was generated by an independent and blinded statistician using a computerised procedure. All participants and investigators remained blinded to treatment until the analyses were completed. After an overnight fast, a triple lumen catheter (adapted from Freka Trelumina, Fresenius Kabi Nederland B.V., Zeist, The Netherlands) was introduced trans-nasally via the stomach into the duodenum, under fluoroscopic guidance. The proximal lumen, with an infusion port positioned in the stomach, was used for administration of the test meal and aspiration of gastric contents. The second lumen, positioned 5 cm distal to the pylorus, was used for continuous perfusion of the inert dilution marker polyethylene glycol (PEG)-3350. The third and distal lumen (positioned at the tip and located 15 cm distal to the pylorus) was used for aspiration of duodenal contents. The catheter position was secured by radiology and regularly checked during the tests by measuring pH of each aspirate. Meals, mixed with acetaminophen (Centrafarm B.V., Etten-Leur, The Netherlands), and either AN-PEP or placebo, were infused into the stomach over a 5-min period, at a rate of 80 mL/min. A PEG-3350 (Norgine B.V., Amsterdam, The Netherlands) solution (15 mg/mL in 0.9% saline) was continuously infused into the proximal part of the duodenum at 3 mL/min to calculate the gluten amount corrected for dilution of duodenal content by endogenous secretions.[27–30] Infusion started 60 min prior to meal infusion, to achieve steady-state conditions in fluid secretion and absorption at the start of meal infusion, and continued till 240 min. Gastric and duodenal content was sampled at baseline ($t = 0$ min), and after start of meal infusion at $t = 15, 30, 45, 60, 75, 90, 120, 150, 180, 210$ and 240 min. Gastric content was also aspirated at $t = 5$ and $t = 10$ min. Mixing of the meal with AN-PEP or placebo and acetaminophen was performed immediately prior to the start of infusion. Approximately 3 mL and 2 mL were aspirated from the gastric and duodenal port, respectively, for pH and gluten epitope measurements. Also, acetaminophen concentrations in gastric samples were measured. The samples were immediately frozen in liquid nitrogen to stop enzyme activity and were subsequently stored at -80 °C until analysis.

Gluten meals

Dry meals were packaged in sachets of airtight tinfoil and stored at room temperature. On test days, meals were prepared at a food-grade kitchen facility. During preparation of the test meal, 1 g acetaminophen was added. Table 1 shows the composition of the meals. All test meals contained 5.2 g of gluten powder, of which 4.0 g was gluten protein (Syral, Aalst, Belgium). Encapsulated refined olive oil powder (VanaGrasa 80B; FrieslandCampina, Kievit B.V., Meppel, The Netherlands) was used as fat source for the meals and together with maltodextrin as additional energy source for the high calorie meal, and sodium caseinate (DMV, Veghel, The Netherlands) was used to match both meals for protein content. The dry meal powders were dissolved in a total volume of 300 mL tap water of 40 °C by stirring with a spoon and subsequently mixing with a Turrax (Ultra Turrax T25; IKA, Staufen, Germany). Low and high caloric meals were not blinded to the investigator.

AN-PEP enzyme and placebo

The AN-PEP enzyme was obtained from DSM Food Specialties (Delft, The Netherlands). A total of 6.1 mL of AN-PEP corresponding with 1.600.000 Protease Picomol International (1 Protease Picomol International is the amount of enzyme that releases one picomole of p-nitroaniline per second under defined assay conditions) in a total of 100 mL water was added to the 300 mL test meals. A 6.1 mL solution consisting of 4.8 g water, 1.3 g maltodextrin, 0.01 g caramel liquid (Brenntag, Deerlijk, Belgium), 0.03 g citric acid and 0.02 g sodium benzoate (Wuhan Youji, Wuhan, China) at pH 4.2, with a similar appearance to AN-PEP, served as placebo.

Sample pre-treatment

Upon thawing, the enzyme in the gastric and duodenal samples was immediately inactivated by increasing the pH of the sample to 11–12 using 1 mol/L NaOH, heating at 85 °C for 10 min and neutralising the pH with 1 mol/L HCl. For the gluten content analysis, 100 μL from each sample was frozen again at -80 °C for further analysis by ELISA, or mixed with loading buffer (60% glycerol, 300 mmol/L Tris, pH 6.8, 12 mmol/L EDTA, pH 8.0, 12% sodium dodecylsulphate, 864 mmol/L 2-mercaptoethanol, 0.05% bromophenol blue), boiled for 5 min for further analysis by Coomassie Blue staining and western blot.

Gluten monitoring by ELISA analyses

The sample was diluted 40–5000 times in phosphate-buffered saline and the presence of the DQ2.5-glia-α3 epitope was quantified using the Gluten-Tec ELISA assay

Table 1 | Recipe and nutritional composition of the low and high calorie test meal per 300 g portion

	Low calorie meal	High calorie meal
Vana Grasa (g)	7.0	22.0
Sucrose (g)	17.0	16.9
Maltodextrin (g)	0.0	36.3
Caseinate (g)	0.7	0.3
Gluten powder (g)	5.2	5.2
Citric acid (g)	0.1	0.1
Water (g)	270.9	245.1
Protein (g)	4.9	4.9
Fat (g)	5.7	17.7
Carbohydrate (g)	18.0	56.5
Ash (g)	0.2	0.5
Protein (kcal)	19.6	19.6
Fat (kcal)	51.6	159.6
Carbohydrate (kcal)	71.9	225.8
Total (kcal)	143.1	405.1
Caloric density (kcal/g)	0.5	1.4
Osmolarity (mOsm/kg)	194.8	373.6
pH	6.0	6.0

(EuroProxima B.V., Arnhem, The Netherlands) according to the manufacturer's instructions.[31] The DQ2.5-glia-α3 epitope is directly adjacent to the 33-mer that contains the immunodominant DQ2.5-glia-α1 and DQ2.5-glia-α2 epitopes. As the α-gliadins contain only a single copy of the DQ2.5-glia-α3 epitope, the measurement of this epitope provides an accurate estimate of the actual α-gliadin content of the samples.[32]

Volume marker

PEG-3350 concentrations were determined in samples obtained from the distal aspiration port in the duodenum, using reversed-phase HPLC in combination with evaporative light scatter detection. The analysis was based on PEG analysis as described by van Wijck *et al.*[33] PEG-3350 concentrations were used to calculate the dilution of duodenal samples by endogenous secretions, using the formula described by Beglinger *et al.*[27]

$$V = (F \times [\text{PEG}]_{\text{perfused}} \times 15)/(\text{PEG}_{\text{measured}})$$

V represents the calculated duodenal volume (mL per 15 or 30 min); F the flow rate of PEG solution perfused (3 mL/min); $[\text{PEG}]_{\text{perfused}}$ the concentration of PEG in the perfusate; $\text{PEG}_{\text{measured}}$ the concentration of PEG in the duodenal juice collected for 15 or 30 min. The number '15' has to be replaced by '30' if the time interval between two samples was 30 min.

To calculate the absolute duodenal gluten amount at a certain time point, the calculated duodenal volume was multiplied with the gluten concentration at that time point.

Gluten monitoring by western blot

Measurement of gluten epitopes by ELISA is an indirect method of gluten analysis. To confirm the presence of intact gluten proteins and relatively large fragments thereof we also performed western blot analysis. The proteins present in samples isolated from the stomach were separated on SDS-PAGE, blotted onto polyvinylidene fluoride membrane and the gliadin proteins were visualised with a monoclonal antibody specific for the immunodominant DQ2.5-glia-α1 epitope.[34]

Presence of AN-PEP in gastric and duodenal samples

To assess the presence of AN-PEP protein in gastric and duodenal samples, the protein in the samples was separated on SDS-PAGE followed by Coomassie Blue staining.

Assessment of gastric emptying

Gastric emptying rate was measured at all test days of 6 of the 12 participants who completed the study, randomly chosen. Gastric emptying rate was determined according to the changes of the acetaminophen concentration over time in the stomach, with stomach samples taken at predetermined time points. Gastric fluid samples were first deproteinated by adding a 10% solution of trichloroacetic acid. After centrifugation (800*g*, 5 min) the supernatant was injected into HPLC using a reversed-phase method with UV detection at 250 nm (Agilent 1100 series HPLC Value System, Waldbronn, Germany). A composition of MilliQ and acetonitrile (97: 3%v/v) was used to elute the samples. As the total gastric volume changes constantly after meal ingestion, it was difficult to calculate the concentration via a formula. A calibration curve of acetaminophen was used to calculate the acetaminophen concentration in the samples. As a pragmatic approach, the total gastric emptying time (in minutes) was derived from this acetaminophen concentration-time curve. The time point when the acetaminophen concentration in a gastric sample is zero indicates the complete passage of the test meal into the duodenum and is thus considered to represent total gastric emptying time.

Gastrointestinal symptoms questionnaire

At the end of each test day, participants were requested to complete a 'Symptoms Diary', to ensure that all gastrointestinal complaints of the test day, caused by the intervention, were reported to the investigator. This

questionnaire included eight items, each rated on a five-point Likert scale. The lowest score, 1, denotes no symptoms and 5 denotes the most pronounced symptoms. Items that were included in the 'Symptoms Diary' were: abdominal discomfort, abdominal pain, abdominal distension, constipation, diarrhoea, flatus, eructation and nausea.

Statistics

The primary outcome of this study was the effect of AN-PEP on gluten degradation, measured by the difference in the 240-min AUC ($AUC_{0-240 \, min}$) of duodenal gluten epitope concentration between AN-PEP and placebo. Secondly, we investigated the effect of AN-PEP on gluten degradation, measured by the difference in $AUC_{0-240 \, min}$ of absolute amounts of gluten epitopes in the duodenum between both interventions. The $AUC_{0-240 \, min}$ was calculated by using the trapezoidal equation. We calculated that a sample size of 12 subjects would be required based on a standardised effect size of 1.3, a power of 90% and $\alpha = 0.05$ (one sided). Seventeen subjects were recruited taking into account a drop out of five subjects. Baseline characteristics are presented as mean (s.d.) for numerical variables and number (%) for categorical variables. Differences in $AUCs$, gastric emptying rate and 'Symptom Diary' scores between combinations of treatment (AN-PEP or placebo) and meal (high or low calorie) were assessed using linear mixed models based on restricted maximum likelihood, where the natural logarithm of the $AUCs$ was taken into account for the expected non-normality. The linear mixed model accounts for the correlation between repeated measures within a person (cross-over design) and missing data, where a likelihood approach was used assuming missing data at random. Fixed factors were treatment, meal, treatment × meal and test day. The best fitting covariance structure, i.e. structure of variances over different test days and correlations between test days, was based on Akaike's Information Criterion. All statistical analyses were performed using IBM SPSS Statistics for Windows (Version 20.0; IBM Corp., Armonk, NY, USA). Two-sided $P \leq 0.05$ were considered statistically significant.

RESULTS

Study subjects

A total of 12 healthy volunteers [67% male; age 26 ± 6 years; BMI (in kg/m^2) = 22 ± 3] were included in the study. One of these subjects did not complete the fourth test day and only the results of the two high

calorie meal test days were available for inclusion in the analyses. In two other subjects the catheter progressed more distally into the small intestine on one occasion, causing administration of (part of) the meal directly into the duodenum. Data of these experiments were omitted, but data from their remaining test days were still included in the analysis as the linear mixed model accounts for missing data. Initially 17 subjects were enrolled in the study, but five subjects dropped out due to discomfort related to the nasoduodenal tube. Data of drop outs were omitted from analyses.

pH of gastric samples

The mean gastric pH of gastric samples, taken during test days when low and high calorie meals combined with AN-PEP were infused, ranged between 2.3 and 5.3 and was similar to the pH range of the samples of placebo-containing meals (data not shown).

DQ2.5-glia-α3 concentrations in stomach and duodenum and absolute amount in duodenum

The mean DQ2.5-glia-α3 concentrations in stomach and duodenum samples after ingestion of low and high calorie meals with and without AN-PEP are shown in Figure 1 and the $AUC_{0-240 \, min}$ in Table 2. The mean duodenal DQ2.5-glia-α3 concentrations per participant are shown in Figure 2. Over a 240-min period, AN-PEP reduced the gastric DQ2.5-glia-α3 concentrations, compared to placebo, both after ingestion of the low (35 vs. 389 μg × min/mL; $P < 0.001$) and the high (53 vs. 386 μg × min/mL; $P < 0.001$; Figure 1a,b; Table 2) calorie meals. This was also observed for duodenal DQ2.5-glia-α3 concentrations (low calorie: 7 vs. 168 μg × min/mL; high calorie: 4 vs. 32 μg × min/mL; $P < 0.001$; Figure 1c,d; Table 2). In the placebo intervention, the duodenal DQ2.5-glia-α3 concentrations were significantly lower after intake of a high compared to a low calorie meal (32 vs. 168 μg × min/mL; $P = 0.001$; Figure 1c,d; Table 2). In the presence of AN-PEP this difference was not present (4 vs. 7 μg × min/mL; $P > 0.05$; Figure 1c, d; Table 2) and low duodenal DQ2.5-glia-α3 concentrations were observed after intake of both a high and low calorie meal. The $AUC_{0-240 \, min}$ of DQ2.5-glia-α3 concentrations in the duodenum of AN-PEP-receiving subjects was around or lower than the limit of detection (26.7 μg/L × 240 min = 7 μg × min/mL) and lower than the limit of quantification (89 μg/L × 240 min = 21 mg × min/L) of the ELISA assay. The pattern for the duodenal DQ2.5-glia-α3 concentrations corresponds with the data for absolute duodenal
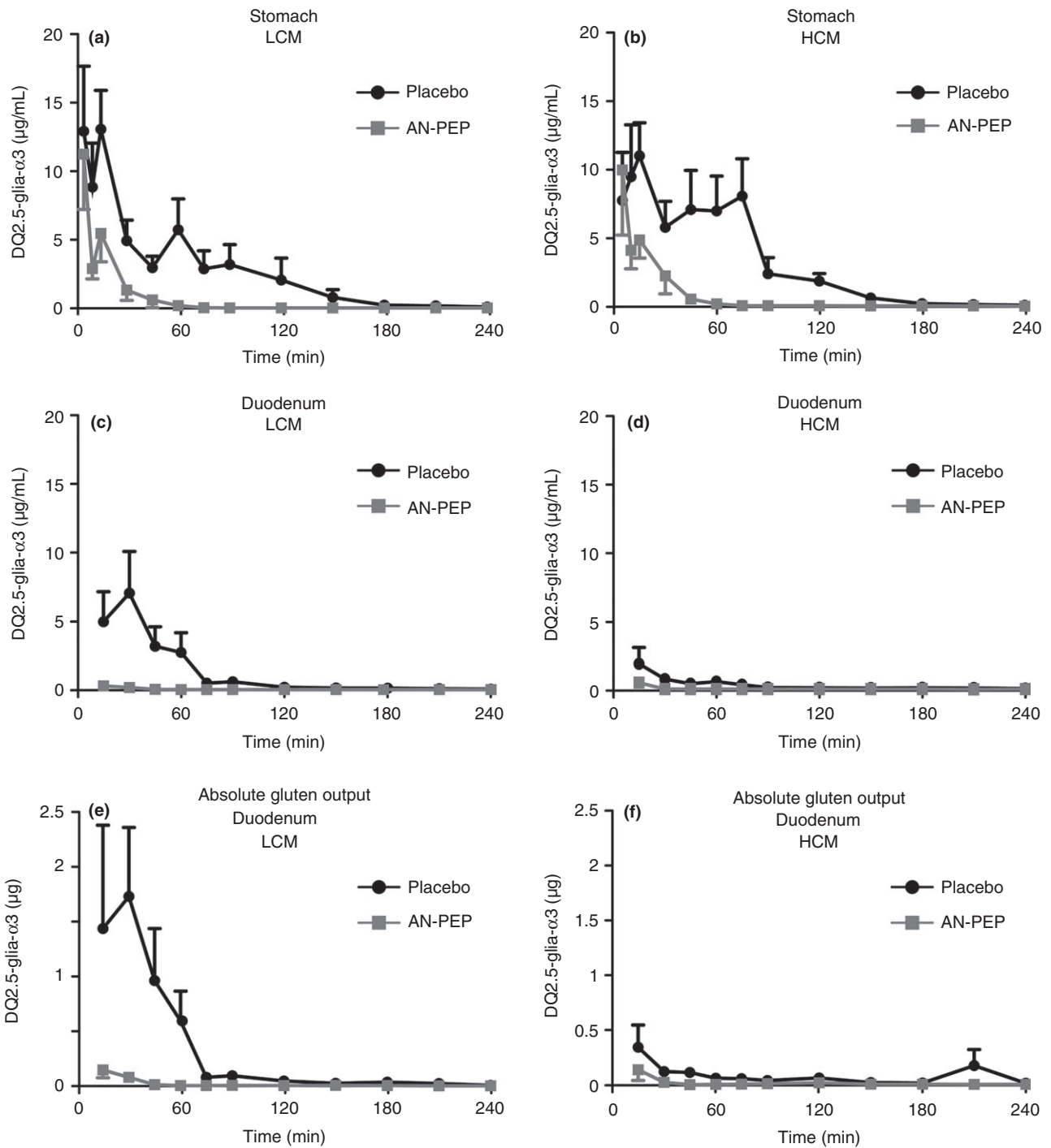
Figure 1 | (a) DQ2.5-glia-α3 concentration (mean ± SEM) over time in the stomach in low calorie meals. (b) DQ2.5-glia-α3 concentration (mean ± S.E.M.) over time in the stomach in high calorie meals. (c) DQ2.5-glia-α3 concentration (mean ± S.E.M.) over time in the duodenum in low calorie meals. (d) DQ2.5-glia-α3 concentration (mean ± S.E.M.) over time in the duodenum in high calorie meals. (e) Absolute DQ2.5-glia-α3 output (mean ± S.E.M.) over time in the duodenum in low calorie meals. (f) Absolute DQ2.5-glia-α3 output (mean ± S.E.M.) over time in the duodenum in high calorie meals.

DQ2.5-glia-α3 amount, which is corrected for the dilution during the digestion process. AN-PEP lowered the calculated absolute duodenal α-gliadin compared to placebo in both low (2813 vs. 31 952 μg × min; $P < 0.001$) and high (2553 vs. 13 095 μg × min; $P = 0.013$; Figure 1e,f; Table 2) calorie meals.

Table 2 | AUC$_{0-240\text{ min}}$ of DQ2.5-glia-α3 concentrations in stomach and duodenum and the AUC$_{0-240\text{ min}}$ of absolute DQ2.5-glia-α3 amount in duodenum

	Low calorie meal		High calorie meal	
	Placebo	AN-PEP	Placebo	AN-PEP
AUC$_{0-240\text{ min}}$ DQ2.5-glia-α3, μg × min/mL				
Stomach				
Mean	389	35*	386	53*
95% CI	180–840	17–73	192–775	25–113
Duodenum				
Mean	168	7*,†	32‡	4*,§
95% CI	80–352	3–14	16–63	2–9
AUC$_{0-240\text{ min}}$ DQ2.5-glia-α3, μg × min				
Duodenum				
Mean	31 952	2813*	13 095	2553¶
95% CI	12 670–80 579	1206–6555	5967–28 730	884–7369

* $P < 0.001$ compared to placebo.

‡ $P = 0.001$ compared to low calorie placebo meal.

¶ $P < 0.05$ compared to placebo.

† Below the level of quantitation 21 μg × min/mL.

§ Below the level of detection 7 μg × min/mL.

Gluten monitoring by western blot

Western blot analysis of stomach samples indicated that gluten degradation was accelerated by the addition of AN-PEP. Compared to samples of placebo-containing meals, samples of AN-PEP-containing meals showed generally a markedly faster degradation of DQ2.5-glia-α1. In many cases, very little or even no gluten protein could be detected when AN-PEP was taken with the meal. In contrast, in gastric samples of placebo-containing meals, in some cases DQ2.5-glia-α1 was still detectable up to 2 h after meal infusion. In duodenal samples, the western blots were unable to detect significant amounts of intact gluten proteins, neither with AN-PEP, nor with placebo (Figure 3). This finding suggests that in both cases little or no intact water-insoluble gluten protein reaches the duodenum.

Overall, for the majority of the meals analysed, the pattern on gluten degradation by both ELISA and western blot showed a correlation, demonstrating the robustness of the study results.

Presence of AN-PEP in gastric and duodenal samples

After administration of AN-PEP-containing meals, a band with AN-PEP's characteristic molecular weight (66 kD) was visible in the gastric samples of 14 different test days (Figure 4). In the other test days, the AN-PEP signal was either too weak to be detected or masked by other proteins with a similar electrophoretic mobility. In the placebo-containing meals, a band with this particular molecular weight was always absent (data not shown). With high calorie meals, AN-PEP was detectable for a longer period than with low calorie meals. AN-PEP was not found in the duodenal samples of any test day (Figure 4), possibly due to degradation of the enzyme by trypsin or chymotrypsin under conditions of high pH in the duodenum.

Gastric emptying

The mean of total gastric emptying time of the high calorie meals was approximately twice as long as compared to the low calorie meals, being significantly different in the presence of placebo (172 vs. 88 min; $P = 0.014$) but not in the presence of AN-PEP (154 vs. 100 min; $P = 0.100$).

Gastrointestinal symptoms questionnaire

Mild gastrointestinal symptoms were reported on some occasions during low and high calorie meal intervention. Overall, discomfort was low for each different intervention. No significant differences were observed in reported gastrointestinal symptoms between meal types in combination with AN-PEP or placebo (data not shown).

DISCUSSION

In the current placebo-controlled intervention study, AN-PEP-mediated gluten digestion was studied in the

stomach and duodenum of healthy volunteers. This is the first study showing that the AN-PEP enzyme efficiently degrades gluten from a meal in the stomach of human subjects.

Considering the enzyme's optimum pH range between 3 and 5, and the mean pH of the gastric samples ranged between 2.3 and 5.3 with both low and high calorie meals, this points to optimal enzyme activity during the entire digestive process in the stomach.[19] Irrespective of the caloric density of the meal, the enzyme degraded almost all the gluten present in the stomach within a period of 1 h, whereas with placebo, gluten were present for 3 h. Furthermore, the addition of AN-PEP did not result in differences in gastrointestinal-related symptoms compared to placebo, confirming that intake of the enzyme is safe and well tolerated by human subjects.[26]

We also tested whether increasing meal caloric density would improve gluten degradation by delaying gastric emptying and thus prolonging exposure time of gluten proteins to AN-PEP and endogenous gastric proteases. Although the low calorie meal in the stomach was emptied within about 1.5 h, the gastric emptying of the high calorie meal took about twice as long. Delayed gastric emptying also resulted in longer gastric residence time of AN-PEP. These findings are consistent with a previously reported delay in gastric emptying rate with increased meal caloric density in human subjects.[35] Within 1 h, AN-PEP degraded gluten to concentrations around or below reliably detectable levels, irrespective of meal caloric density. Without AN-PEP enzyme, high gluten concentrations were present in the stomach when given with a low or high calorie meal, supporting the notion that pepsin exerts only minimal proteolytic action on dietary gluten.[36] Interestingly, in absence of the enzyme, less gluten reached the duodenum with high than with low calorie meals. Possibly, the fat content of the high calorie meals supported gluten digestion in the duodenum by increasing pancreatic enzyme outputs which has been described for high-fat as compared to low-fat diets.[37]

We used a marker infusion technique to correct for dilutions resulting from biliary and pancreatic secretions that might have influenced the gluten concentrations measured in the duodenum. The absolute gluten values obtained, therefore, represent a better measure of true gluten exposure than the gluten concentrations. The absolute amount of gluten reaching the duodenum was significantly reduced with AN-PEP irrespective of meal caloric density, consistent with the findings for gluten concentrations. The duodenal gluten degradation pattern was comparable between concentrations and absolute amounts suggesting little influence of duodenal dilutions. Insoluble gluten measurements confirmed that AN-PEP is able to significantly reduce gluten before entering the duodenum.

A band on SDS-PAGE with AN-PEPs characteristic molecular weight was observed in gastric, but not in duodenal aspirates, indicating AN-PEP is present and active in the stomach but not in the duodenum. Possibly, under duodenal neutral pH conditions, bile and pancreatic enzymes may have degraded the enzyme.

Apart from AN-PEP, other enzymes detoxifying gluten are currently under investigation. A mixture of two proteases, namely PEP derived from *Sphingomonas capsulate* and a barley protease (EP-B2), termed ALV003, has previously been shown to be capable of degrading complex gluten proteins *in vitro*.[20, 23] In a human setting, ALV003 was well tolerated and effective in detoxifying 1 g of gluten.[21] A recent study in coeliac patients showed that ALV003 attenuated small intestinal mucosal injury induced by 6-week ingestion of 2 g gluten daily.[22] Another protease mix, STAN-1, showed effective *in vitro* gluten-degrading properties.[38] These enzymes have been investigated for their applicability as a future coeliac disease drug therapy.

This study made use of a triple lumen nasogastroduodenal catheter. This enabled the simultaneous administration of a test meal, infusion of a dilution marker, and aspiration of gastric and duodenal contents. Clear benefit of this approach is that it allowed us to measure the actual gluten concentration present in duodenum samples. This information is important for safe use in subjects intolerant to gluten. Further, by infusion of the dilution marker we could calculate the absolute intraduodenal gluten appearance. In none of previous mentioned studies, investigating other gluten-detoxifying enzymes, true gluten presence in the duodenum has been measured. To standardise each meal intake, AN-PEP was added to the meal and thereafter immediately infused intragastrically at a standardised rate, to avoid differences in gluten degradation during meal consumption between interventions, caused by variable meal ingestion rates. We acknowledge that this does not represent a fully physiological meal setting, in which solid food and AN-PEP are ingested separately and undergo the normal physiological processes of mixing in the stomach. A randomised placebo-controlled trial is underway in which the efficacy of AN-PEP in an actual meal setting will be investigated. Furthermore, this technique also has another drawback. Migration of the catheter, due to gastrointestinal peristalsis, caused erroneous infusion of the
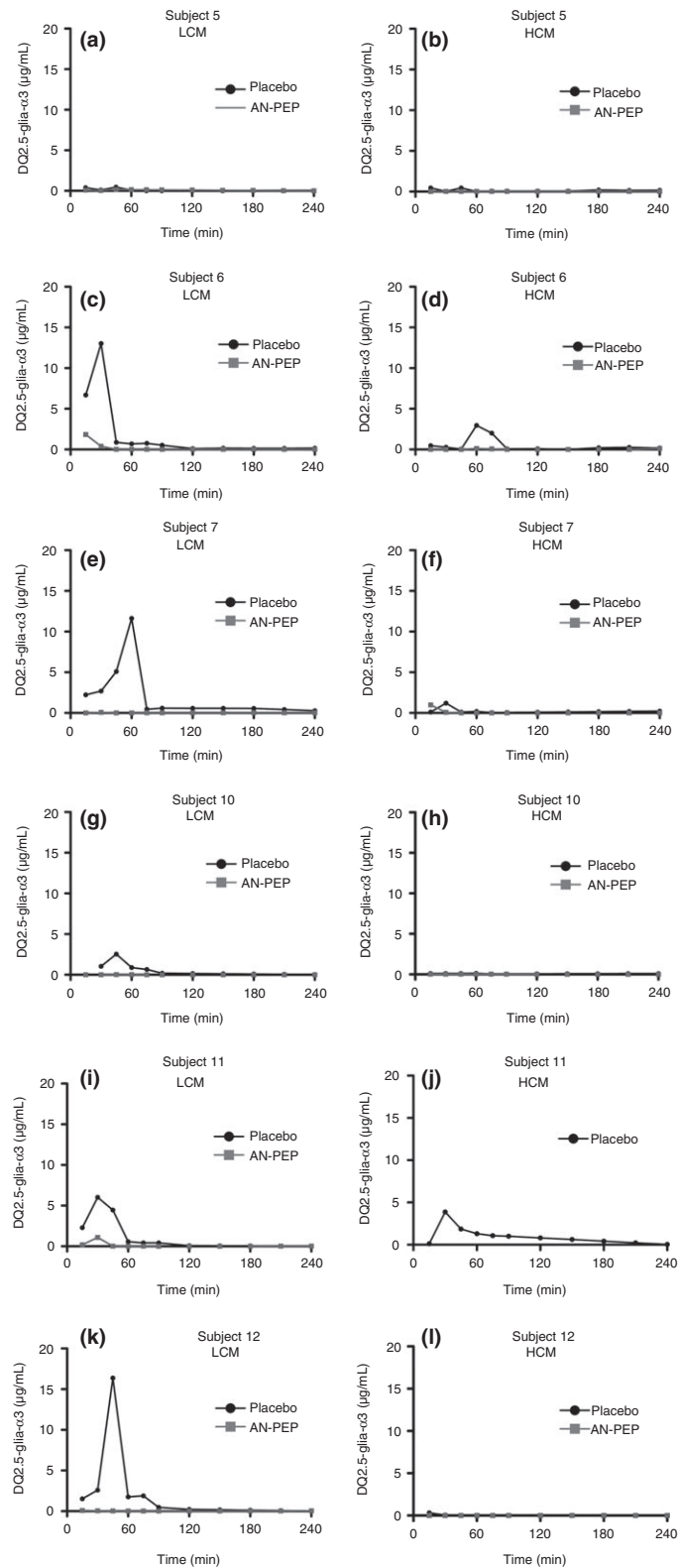
Figure 2 | DQ2.5-glia-α3 concentration over time in the duodenum of the individual subjects in low calorie meals (LCM) and high calorie meals (HCM). (a) + (b): subject 5. (c) + (d): subject 6. (e) + (f): subject 7. (g) + (h): subject 10. (i) + (j): subject 11. (k) + (l): subject 12. (m) + (n): subject 13. (o) + (p): subject 14. (q) + (r): subject 15. (s) + (t): subject 16. (u): subject 17. (v) + (w): subject 19.
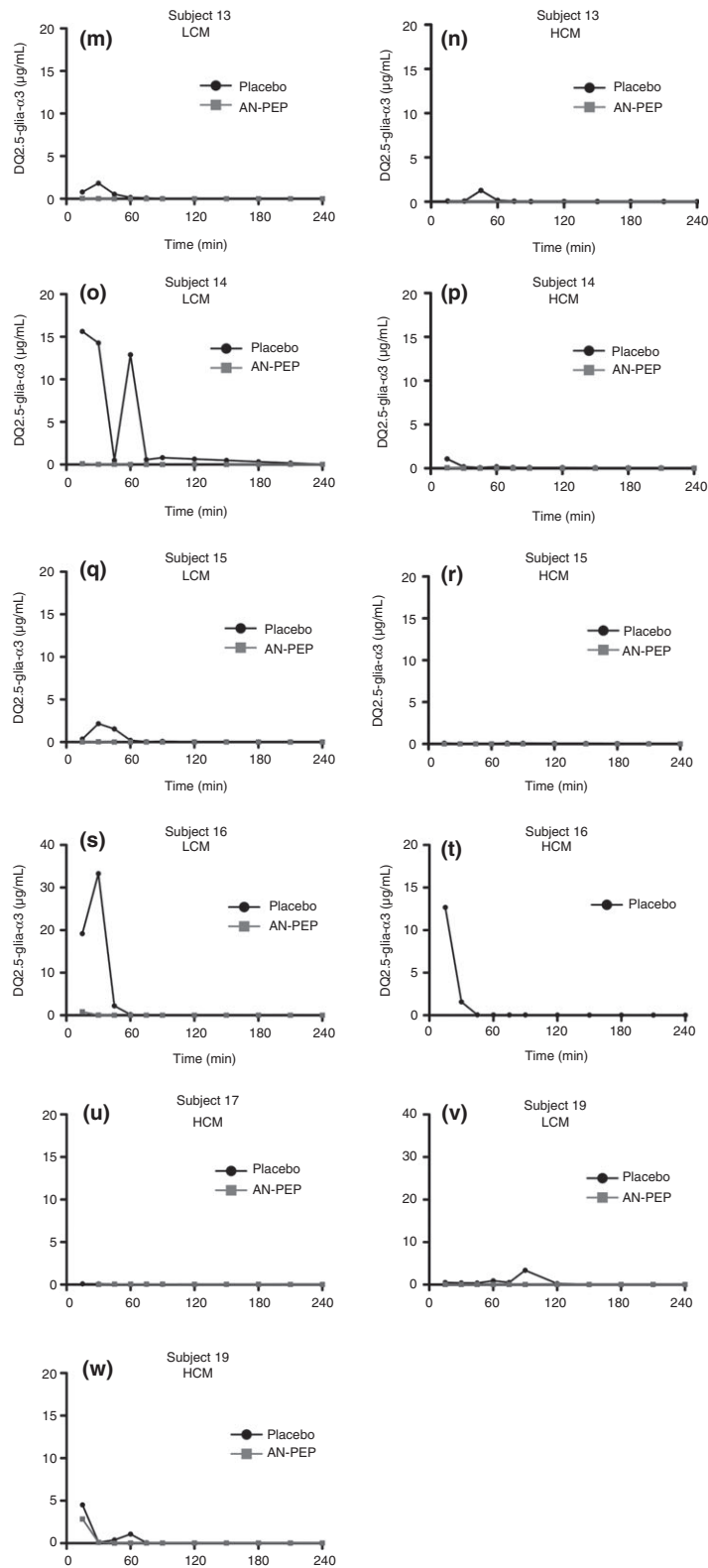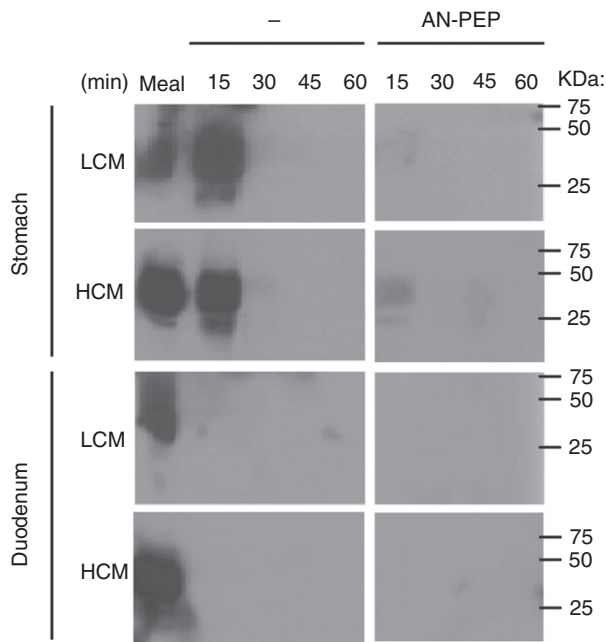
Figure 2 | continued

meal into the duodenum in two subjects at one occasion, as was noted based on pH profiles. These data were excluded from analysis.

The AN-PEP enzyme has been developed as a dietary supplement that in conjunction with a gluten-free diet may help subjects intolerant to gluten to digest unin-

Figure 3 | Representative western blot showing degradation of water-insoluble DQ2.5-glia-α1 over time in the stomach and duodenum in low and high calorie meals with and without AN-PEP.
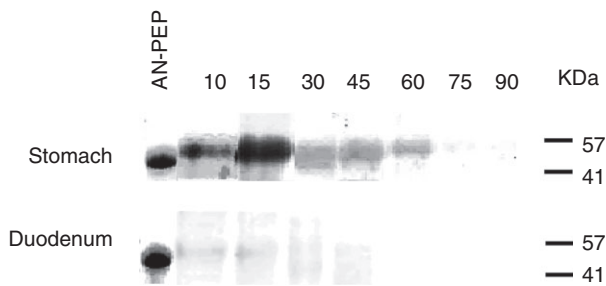
Figure 4 | Representative SDS-PAGE gel showing the presence of AN-PEP protein in gastric and duodenal aspirates.

In conclusion, AN-PEP efficiently degrades gluten from a meal in the stomach of healthy volunteers before entering the duodenum. Increasing the caloric density of a gluten meal slowed gastric emptying rate and prolonged gastric residence time of the enzyme. Since AN-PEP with a low calorie meal already degraded almost all gluten, a high calorie meal could not further increase the efficacy of the enzyme to digest gluten.

AUTHORSHIP

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tended dietary gluten. Despite these promising results, the data do not prove that AN-PEP allows subjects intolerant to gluten to ingest gluten safely. Oral enzymes cannot replace a gluten-free diet, yet our observations suggest that AN-PEP may be useful as a digestive aid to help digest hidden gluten. The enzyme may protect against unintentional ingestion of gluten on a daily basis, during social events or travelling. A randomised placebo-controlled trial is underway to address AN-PEP's efficacy in the target population which is necessary prior to AN-PEP's use to be considered safe and effective.

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