LEADING ARTICLE

Oral proteases: a new approach to managing coeliac disease

Nadine Cerf-Bensussan, Tamara Matysiak-Budnik, Christophe Cellier, Martine Heyman

A life-long but constraining gluten-free diet is the only treatment currently available for coeliac disease. The human gastrointestinal tract does not possess the enzymatic equipment to efficiently cleave the gluten-derived proline-rich peptides driving the abnormal immune intestinal response in patients with coeliac disease. Oral therapy by exogenous prolylendopeptidases able to digest ingested gluten was therefore propounded as an alternative treatment to the diet. The feasibility of this approach is discussed by reviewing recent data on the intestinal transport of gliadin peptides, properties of available enzymes and preliminary clinical assays. Development of new enzymes or enzymatic cocktails offers potentially more potent therapeutic tools that, however, need meticulous evaluation based on clinical, biological and histological criteria.

Ever since Dicke's discovery in the 1940s that coeliac disease is caused by wheat and related cereals (gluten), following a life-long, strict exclusion diet has allowed regression of intestinal inflammation, relief of symptoms and prevention of most complications. Gluten is however a widespread (and in many countries unlabelled) ingredient in the human diet. Bona fide gluten-free products are not widely available and are more expensive than their gluten-containing counterparts.

Gluten-free diet, although an efficient and safe treatment, is therefore very constraining, resulting in social burden and poor compliance, and warranting the search for therapeutic alternatives.

Toxic proteins for patients with coeliac disease, collectively named gluten, are present in the non-hydrosoluble fraction of flour from wheat, barley and rye. Their name—prolamines—stems from their high content in proline and glutamine residues, a composition that underlies their role in bread making but promotes their resistance to digestive enzymes and their recognition by the immune system. Wheat prolamines, the best characterised, comprise several hundred proteins divided into monomeric α -gliadins, γ -gliadins and ω -gliadins, and polymeric glutenins. Analysis of their toxicity suggests that harmful peptides can be divided into two groups acting on the two arms of immunity. A first set of studies, initiated by the identification of HLA-DQ 2/8 as the major genetic risk factor in coeliac disease, have provided

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definitive evidence that the toxicity of prolamines largely relies on their capacity to trigger a specific immune response in the intestine. After their deamidation by tissue transglutaminase 2 (tTG), many gluten peptides can bind the HLA-DQ2 and HLA-DQ8 molecules expressed by antigen-presenting cells and activate gliadin-specific CD4+ intestinal T lymphocytes that release the proinflammatory cytokine interferon (IFN) γ .² A second set of more recent studies suggests that other gluten-derived peptides exert toxic effects on the intestinal mucosa independently of their specific recognition by T lymphocytes. The most studied is peptide 31–43/49, common to the N-terminus of α -gliadins. This peptide can, via unknown signalling pathways, induce the production of interleukin 15 (IL15).^{3,4} This proinflammatory cytokine, massively increased in active coeliac disease, drives the expansion of intraepithelial lymphocytes, licenses them to kill epithelial cells via innate immune receptors and promotes the emergence of lymphomas.^{3,5,6}

Increased understanding of the molecular basis of coeliac disease has led to the possibility of new targets in the treatment of this disease.⁷ Potential approaches include:

- block deamidation of gluten peptides by tTG,
- or their binding to HLA-DQ2/8,
- or to silence gluten-reactive T cells by immunotherapy.

Yet, the feasibility and safety of these approaches remain uncertain, inasmuch as there is not yet a good animal model of coeliac disease to design preclinical studies. Humanised antibodies have recently been developed against IFN γ and IL15, two cytokines with a putative prominent role in intestinal damage in coeliac disease. Although the anti-IL15 antibody might be valuable in refractory sprue to block the expansion and activation of clonal intraepithelial lymphocytes that are characteristic of this severe and currently intractable condition,^{3,5} the possible benefit of such drugs in uncomplicated coeliac disease remains as yet largely outweighed by the unacceptable risk of severe side effects.

The most advanced therapeutic proposal, however, and the only one which has led to preliminary clinical studies is the use of oral proteases to help degrade toxic gliadin peptides before they reach the mucosa.

Abbreviations: PEP, prolylendopeptidase; tTG, tissue transglutaminase 2

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This proposal resurrects the "missing peptidase" hypothesis propounded in the 1960s by Frazer, who had observed that gliadin could be detoxified with an extract of pig intestinal mucosa.⁸ Following this observation, several studies investigated a possible enzymatic defect in the intestinal mucosa of patients with coeliac disease. Conflicting results were reported on a possible primary or secondary defect in brush border peptidases,^{9–10} but no genetic polymorphisms could be detected in dipeptidyl peptidase IV, aminopeptidase N¹¹ or prolylendopeptidase (PEP).¹² Our own results obtained with biopsies mounted in Ussing chambers suggest reduced activity of brush border enzymes both in active and treated coeliac disease, but full epithelial recovery in treated coeliac disease is difficult to ascertain.¹³

Although clear-cut evidence of a constitutive enzyme defect specific for coeliac disease is still lacking, recent elegant studies by Shan *et al* indicate that the lack of endoprolol peptidase activity in gastric and pancreatic enzymes, and in the human intestinal brush border, prevents efficient enzymatic attack of proline-rich domains in gluten proteins.¹⁴ This inefficient digestion promotes the release, at the mucosal surface, of large peptides endowed with potent immunostimulatory properties. These peptides may cross the epithelium and reach the intestinal lamina propria in amounts sufficient to trigger the activation of CD4+ T cells in at-risk individuals. Thus, using recombinant α 2-gliadins and more recently γ 5-gliadins as model proteins, these authors showed that digestion by luminal enzymes yielded large peptides of, respectively, 33 and 26 amino acids, each containing several major T cell epitopes.^{14–15} Both peptides were excellent substrates for tTG and 3–30 times more potent than the corresponding single epitopes at stimulating in vitro proliferation of gliadin-specific intestinal T cells.^{14–15} The 33 mer peptide was also highly resistant to proteolysis by brush border enzymes.¹⁴ Moreover, α 2-gliadin deleted from the 33 mer failed to stimulate gliadin-specific T cells, suggesting that the cluster of T cell epitopes in the 33 mer was the sole source of immunogenicity in this protein.¹⁵ Further computational analysis of these experimental data indicated that clustering of known or putative T cell epitopes in proline-rich regions predicted to be highly resistant to luminal proteolysis is a characteristic feature of prolamines toxic for patients with coeliac disease that is absent in non-toxic dietary proteins.^{15–16}

These results led Koshla, Sollid and coworkers to test the hypothesis that an exogenous PEP derived from *Flavobacterium meningosepticum* might help to digest and thereby detoxify gliadin peptides. They showed that addition of PEP either in vitro in the presence of brush border extracts or during in vivo perfusion of rat small intestine resulted in extensive breakdown of the 33 mer peptide and concomitant loss of its capacity to stimulate gliadin-specific T cells. This confirmed that abundance or location of proline residues is a crucial factor contributing both to resistance to luminal proteolysis and to immunogenicity of gliadin peptides.¹⁴ This experimental result provided a strong rationale to propound oral proteases as an alternative therapy in coeliac disease. The enzymes could be administered at a time of a meal so that they are released or activated in the upper gastrointestinal lumen, where they could complement gastric and pancreatic enzymes in detoxification ingested gluten and prevent harmful peptides from reaching the mucosal surface. This proposal is very attractive as oral proteases have been used for many years to treat pancreatic insufficiencies efficaciously and with no side effects.

A preliminary double-blind crossover study was recently performed by Gray, Koshla *et al* in a small number of asymptomatic patients with coeliac disease to assess the capacity of the recombinant PEP from *F meningosepticum* to

efficiently detoxify 5 g of gluten (the equivalent of a slice of bread).¹⁷

Two-week oral challenges separated by a washout period were performed with orange juice added with 5 g gluten that was either left undigested or digested for 1 h with 200 U PEP/g gluten. The effect of the challenge was assessed by measuring D-xylose absorption and faecal fat excretion. The high prevalence of abnormal absorptive functions at the baseline in these patients (who did not have a control histological examination before entering the study) was a major drawback, and positive challenge by undigested gluten but not by PEP-digested gluten was shown only in a small number of patients. Although these results might suggest some protection, they remain too limited to draw any definite conclusion. Furthermore, the fact that some patients might respond to a PEP digest that had apparently lost its immunogenicity when tested in vitro on gliadin-specific T cell lines remains puzzling.¹⁷ A second clinical assay based on oral enzyme therapy has recently been published by Cornell *et al*.¹⁰ These authors treated 21 patients with histologically proved, clinically silent coeliac disease, with undefined enzyme animal extracts after oral challenge with gluten. They concluded that there was a potential benefit of enzyme therapy as compared with placebo. Yet, the lack of consistency of histological changes in the few tested patients (most of whom had histological changes before the assay) and the modest changes in anti-tTG antibodies preclude, in our view, drawing any definite conclusions. The results of these two studies underscore the incidence of functional and histological changes in patients with coeliac disease who are prescribed a strict gluten-free diet and the resulting difficulties in evaluating properly the effect of alternative therapies. They emphasise the need for complementary approaches to evaluate their pertinence and feasibility.

One important question that remains is whether the properties of the tested PEP are adequate to promote efficient digestion of gluten in the duodenal lumen, and whether this digestion could efficiently prevent the abnormal transport of toxic peptides across the intestinal epithelium in patients with coeliac disease. In vitro analysis of the efficacy of PEP from *F meningosepticum* on the digestion of the 33 mer and 31–49 peptides indicated that relatively high concentrations of PEP (100–500 U/ml for 200 μ g/ml of peptide) or prolonged exposure was necessary to achieve complete digestion and prevent the intestinal transport of toxic or immunostimulatory fragments.¹⁸ On the basis of in vitro digestion experiments and perfusion experiments on rat small intestine, Shan *et al* have suggested that, in vivo, PEP cooperates with brush border enzymes to accelerate the breakdown of gliadin peptides.¹⁴ We could not show a comparable effect when PEP was added on to the mucosal side of intestinal biopsy specimens (from patients with active coeliac disease) mounted in Ussing chambers. This negative result was probably due to decreased activity of brush border enzymes.¹³ In contrast, we observed that the defect in intraluminal processing of proline-rich peptides could be largely overcome, at least in controls and patients treated for coeliac disease, during their intestinal transport.¹³

Previous studies indicate that large peptides, as proteins, do not leak along the paracellular pathway, but are transported across enterocytes by non-specific transcytosis. This transcellular pathway comprises a minor direct road along which a very small fraction (<10%) escapes degradation and a major degradation pathway (>90%) through the acidic endosomal-lysosomal compartment of enterocytes. Ex vivo experiments with duodenal biopsy specimens mounted in Ussing chambers showed that the 33 mer and 31–49 peptides, although resistant to proteolysis by brush border peptidases, were almost totally degraded during intestinal transport (~90%) in healthy

subjects, indicating that the tested peptides follow the transcytotic route. Comparable results were obtained in patients treated for coeliac disease.¹³ Not surprisingly, adding exogenous PEP into the mucosal compartment had no detectable effect on peptide transport and processing.¹⁸ Incomplete luminal hydrolysis of proline-rich peptides may thus be compensated by epithelial processing in controls and patients treated for coeliac disease. However, in the latter the tiny amount of peptides left undigested after transepithelial transport might reach the threshold of immune reactivity.¹³ In these patients, bacterial PEP, despite its limited efficacy, might help decrease the entrance of toxic peptides under this threshold. Yet, protection by PEP may depend on the individual sensitivity of patients to gluten. Recent evidence suggests that peptide 31–49 might exert some of its toxicity at the epithelial cell surface before transport.

The study of transport and processing of gliadin peptides in biopsy specimens mounted in Ussing chambers suggested that oral therapy by PEP might be even “trickier” in patients with active coeliac disease.

In patients with active coeliac disease, despite the known changes of tight junctions shown by a decrease in electrical resistance of duodenal biopsy specimens, there was no detectable paracellular leakage of peptides. Intestinal transport and processing of peptides were, however, profoundly changed.¹³ Up to 50% of peptide 31–49 or of the 33 mer placed on the mucosal surface was recovered in the serosal compartment either intact or as partially digested peptides with a size corresponding to toxic or immunogenic peptides. In fact, in active coeliac disease, peptide transport seems modified from a non-specific transcellular pathway to a “protected” transport allowing escape from lysosomal enzymes.^{13 18 19} Thus, in active coeliac disease, oral therapy by bacterial PEP will have to overcome both the limited amount of brush border enzymes due to villous atrophy and the protected transepithelial transport of gliadin peptides. Accordingly, it was necessary to add relatively high concentrations of PEP (500 U/ml) in the mucosal compartment of Ussing chambers to prevent the passage of potentially toxic metabolites into the serosal compartment.

Concern about the capacity of PEP derived from *F meningosepticum* to efficiently detoxify gluten in the duodenal lumen has recently led two groups to investigate new enzymes or combination enzyme therapies. Koning and coworkers showed that a newly identified PEP produced by *Aspergillus niger* not only hydrolysed gliadin peptides approximately 60 times faster than PEP from *F meningosepticum* but was also active over a much larger range of pH (2–8, optimum 4.5). This enzyme efficiently hydrolysed gluten under in vitro conditions mimicking the stomach. Therefore it might be useful to reduce markedly the amount of toxic peptides even before they enter the duodenum.²⁰ Stepniak *et al* have taken a slightly different but parallel approach. They first observed that recombinant EP-B2, a cysteineprotease derived from germinating barley seeds, is activated at acid pH and by pepsin and can efficiently hydrolyse α 2-gliadin in vitro in conditions mimicking the gastric lumen.²¹ They subsequently used a rat model to show that EP-B2 can efficiently digest gluten in the stomach and markedly reduce the delivery of intact gluten in the intestinal lumen.²² Finally, these authors provided in vitro evidence that prior treatment by EP-B2 at low pH for 1 h (estimated time spent by the alimentary bulk in the stomach) promoted subsequent detoxification of food-grade gluten by PEP in the presence of a cocktail of pancreatic enzymes. In the in vitro conditions used (gluten:EP-B2:PEP weight ratio of 75:3:1), only the

“two-enzyme glutenase” treatment was able to fully eliminate the capacity of gluten to stimulate gluten-specific CD4+ T cell lines.²³

In conclusion, despite some current drawbacks discussed above, oral therapy by proteases seems a promising and simple approach that needs to be refined.

The risk of side effects seems to be small. Short-term ex vivo studies in Ussing chambers and recent in vivo studies in rats plead against a toxic effect of PEP or EP-B2 on the mucosa, although this should be confirmed by more prolonged in vivo exposure in animal models. It will also be necessary to prove that the enzymes are not allergenic and do not reach the bloodstream intact. Yet, such side effects have not been reported in patients with pancreatic insufficiency treated with oral proteases. Further development of novel enzymes with enhanced efficiency and the use of enzymatic cocktails should enable more rapid and efficient gliadin breakdown in the gastrointestinal lumen and enhanced protection. Finally, even if oral protease therapy cannot fully replace the gluten-free diet, it might improve quality of life either by protecting highly sensitive patients against “hidden gluten” or by allowing ingestion of occasional quantities of gluten during social events or travel, thereby meeting a strong demand of patients with coeliac disease.

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REFERENCES

- 1 Van de Kamer J, Weijers H, Dicke W. Coeliac disease V. Some experiments on the cause of the harmful effect of wheat gliadin. *Acta Paediatr Scand*, 1953;42:223–31.
- 2 Sollid L. Coeliac disease: dissecting a complex inflammatory disorder. *Nat Rev Immunol* 2002;9:647–55.
- 3 Hue S, Mention JJ, Monteiro RC, *et al*. A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. *Immunity* 2004;21:367–77.
- 4 Maiuri L, Ciacci C, Ricciardelli I, *et al*. Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. *Lancet* 2003;362:30–7.
- 5 Mention JJ, Ben Ahmed M, Begue B, *et al*. Interleukin 15: a key to disrupted intraepithelial lymphocyte homeostasis and lymphomagenesis in celiac disease. *Gastroenterology* 2003;125:730–45.
- 6 Meresse B, Chen Z, Ciszewski C, *et al*. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity* 2004;21:357–66.
- 7 Sollid LM, Khosla C. Future therapeutic options for celiac disease. *Nat Clin Pract Gastroenterol Hepatol* 2005;2:140–7.
- 8 Frazer A, Fletcher R, Ross C, *et al*. Gluten-induced enteropathy: the effect of partially digested gluten. *Lancet* 1959;2:252–5.
- 9 Bruce G, Woodley JF, Swan CH. Breakdown of gliadin peptides by intestinal brush borders from coeliac patients. *Gut* 1984;25:919–24.
- 10 Cornell HJ, Macrae FA, Melny J, *et al*. Enzyme therapy for management of coeliac disease. *Scand J Gastroenterol* 2005;40:1304–12.
- 11 Clot F, Babron MC, Percopo S, *et al*. Study of two ectopeptidases in the susceptibility to celiac disease: two newly identified polymorphisms of dipeptidylpeptidase IV. *J Pediatr Gastroenterol Nutr* 2000;30:464–6.
- 12 Diosdado B, Stepniak DT, Monsuur AJ, *et al*. No genetic association of the human prolyl endopeptidase gene in the Dutch celiac disease population. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G495–500.
- 13 Matysiak-Budnik T, Candall C, Dugave C, *et al*. Alterations of the intestinal transport and processing of gliadin peptides in celiac disease. *Gastroenterology* 2003;125:696–707.
- 14 Shan L, Molberg O, Parrot I, *et al*. Structural basis for gluten intolerance in celiac sprue. *Science* 2002;297:2218–2220.

- 15 **Shan L**, Qiao SW, Arentz-Hansen H, *et al*. Identification and analysis of multivalent proteolytically resistant peptides from gluten: implications for celiac sprue. *J Proteome Res* 2005;**4**:1732–41.
- 16 **Vader LW**, Stepniak DT, Bunnik EM, *et al*. Characterization of cereal toxicity for celiac disease patients based on protein homology in grains. *Gastroenterology* 2003;**125**:1105–13.
- 17 **Pyle GG**, Paaso B, Anderson BE, *et al*. Effect of pretreatment of food gluten with prolyl endopeptidase on gluten-induced malabsorption in celiac sprue. *Clin Gastroenterol Hepatol* 2005;**3**:687–94.
- 18 **Matysiak-Budnik T**, Candali C, Cellier C, *et al*. Limited efficiency of prolyl-endopeptidase in the detoxification of gliadin peptides in celiac disease. *Gastroenterology* 2005;**129**:786–96.
- 19 **Matysiak-Budnik et al**. 2006. submitted for publication.
- 20 **Stepniak D**, Spaenij-Dekking L, Mitea C, *et al*. Highly efficient gluten degradation with a newly identified prolyl endopeptidase: implications for celiac disease. *Am J Physiol Gastrointest Liver Physiol* 2006;**291**:621–9.
- 21 **Bethune MT**, Strop P, Tang Y, *et al*. Heterologous expression, purification, refolding, and structural-functional characterization of EP-B2, a self-activating barley cysteine endopeptidase. *Chem Biol* 2006;**13**:637–47.
- 22 **Gass J**, Vora H, Bethune MT, *et al*. Effect of barley endopeptidase EP-B2 on gluten digestion in the intact rat. *J Pharmacol Exp Ther* 2006;**318**:1178–86.
- 23 **Siegel M**, Bethune MT, Gass J, *et al*. Rational design of combination enzyme therapy for celiac sprue. *Chem Biol* 2006;**13**:649–58.

EDITOR'S QUIZ: GI SNAPSHOT

Chronic abdominal pain in an alcoholic

Robin Spiller, editor

Clinical presentation

A 38-year-old man with alcoholic liver disease was admitted complaining of intermittent right abdominal pain over a 4 month period. He had been admitted twice during the period with a diagnosis of alcoholic pancreatitis. The pain was relieved after bowel rest and administration of a narcotic agent but recurred despite the absence of alcohol. He did not have fever but lost 5 kg in body weight during the period as a result of decreased appetite. Upper gastrointestinal endoscopy and abdominal ultrasound showed no obvious malignancy. A review of previous image studies relating to his case revealed that an unrecognised calcification had occurred over the right abdomen 4 months earlier (fig 1, arrow). Abdominal computed tomography was then performed to evaluate the lesion (fig 2).

Question

What is the diagnosis?

See page 185 for answer

This case is submitted by:

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Figure 1 Kidney-ureter-bladder x ray disclosed a calcified lesion over the right abdomen (arrow).

Figure 2 Abdominal computed tomography confirmed the calcified lesion located in the right subhepatic space.