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Oral proteases: a new approach to managing coeliac disease

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Abbreviations

BB: brush border
CD: coeliac disease
PEP: prolylendopeptidase (from *Flavobacterium meningosepticum)*
tTG: tissue transglutaminase 2

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ABSTRACT

A constraining life-long gluten-free-diet is the only current treatment for celiac 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 coeliac patients. Oral therapy by exogenous prolyl-endopeptidases able to digest ingested gluten was therefore propounded as an alternative treatment to the diet. The feasibility of this approach is discussed by confronting recent data on the intestinal transport of gliadin peptides, properties of available enzymes and preliminary clinical assays. Development of new enzymes or enzymatic cocktail offers potentially more potent therapeutic tools that need however meticulous evaluation based on clinical, biological and histological criteria.

TEXT

Since the discovery by Dicke in the 1940's that coeliac disease (CD) is caused by wheat and related cereals (gluten) [1], a life-long strict exclusion diet permits to cure intestinal inflammation, relieve symptoms and prevent most complications. Gluten is however a widespread (and in many countries unlabeled) 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 celiac patients, collectively named gluten, are present in the non hydro-soluble fraction of flour from wheat, barley and rye. Their name prolamines holds 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 characterized, comprise several hundred proteins divided into monomeric α- γ- 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-DQ2/8 as the major genetic risk factor in CD, have provided definitive evidence that the toxicity of prolamines largely relies on their capacity to trigger a specific immune response in the intestine. Following 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 IFNγ [2]. A second set of more recent studies suggest 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 IL-15 [3, 4]. This proinflammatory cytokine, massively increased in active CD, 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].

Deciphering the molecular basis of CD allows envisage new targets for rationale therapy [7]. Potential approaches might be to 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 CD to design preclinical studies. Humanised antibodies have recently been developed against IFN-γ and IL-15, two cytokines with a putative prominent role in intestinal damage in CD. While anti-IL-15 antibody might be valuable in refractory sprue to block the expansion and activation of clonal IEL that are characteristic of this severe and currently intractable condition [3, 5], the possible benefit of such drugs in uncomplicated CD remains as yet largely out weighted 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. This proposal resurrects the "missing peptidase" hypothesis propounded in 1960's 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 CD patients. Conflicting results were reported on a possible primary or secondary defect in brush border (BB) peptidases [9, 10], but no genetic polymorphisms could be detected in dipeptidyl peptidase IV, aminopeptidase N [11] or prolyl-endopeptidase [12]. Our own results obtained with biopsies mounted in Ussing chambers suggest reduced activity of BB enzymes both in active and treated CD, but full epithelial recovery in treated CD is difficult to ascertain [13].

While clear-cut evidence of a constitutive enzyme defect specific of CD is still lacking, recent elegant studies by Koshla, Sollid and coworkers indicate that the lack of endoprolyl peptidase activity in gastric and pancreatic enzymes, and in the human intestinal BB 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 that 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 α2- and more recently γ5-gliadins as model proteins, these authors showed that digestion by luminal enzymes yielded large peptides of respectively 33 and 26 aminoacids, 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 to stimulate *in vitro* proliferation of gliadin-specific intestinal T cells [14, 15]. The 33 mer peptide was also highly resistant to proteolysis by BB enzymes [14]. Moreover, α2-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]. Computational analysis completed these experimental data and 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 CD patients that is absent in non toxic dietary proteins [15, 16]

These results led Koshla, Sollid and coworkers to test the hypothesis that an exogenous prolylendopeptidase derived from *Flavobacterium meningosepticum* (PEP) might help to digest and thereby detoxify gliadin peptides. They showed that addition of PEP either *in vitro* in the presence of BB 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, confirming that abundance and/or location of proline residues is a crucial factor contributing both to resistance to luminal proteolysis and immunogenicity of gliadin peptides [14]. This experimental result provided a strong rationale to propound oral proteases as an alternative therapy in CD. The enzymes could be administered at a time of a meal so that they are released or activated in the upper gastro-intestinal lumen where they could complement gastric and pancreatic enzymes to detoxify ingested gluten and prevent harmful peptides to reach the mucosal surface. This proposal is very attractive since oral therapy with proteases has already been used for many years to treat pancreatic insufficiencies with efficacy and no side effects.

A preliminary double blind cross over study was recently performed by Gray, Koshla et al in a small number of asymptomatic CD patients to assess the capacity of the recombinant PEP from *F. meningosepticum* to efficiently detoxify 5g 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 5g gluten that was either left undigested or digested for one hour with 200U PEP/g gluten. Impact of challenge was assessed by measuring D-xylose absorption and fecal fat excretion. High prevalence of abnormal absorptive functions at the baseline in these patients (who did not have control histology before entering the study) was a major drawback, and positive challenge by undigested gluten but not by PEP-digested gluten could only be demonstrated in a very 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 and coworkers. These authors treated 21 patients with histologically proven, clinically silent CD patients, with undefined enzyme animal extracts after oral challenge with gluten. They concluded to a potential benefit of enzyme therapy as compared to placebo [10]. Yet, the lack of consistency of histological changes in the few tested patients (most of whom had altered histology before the assay) and the very modest changes in anti-tTG antibodies, preclude, in our view, to draw any definite conclusions. The results of these two studies underscore the frequency of functional and histological alterations in CD patients who are thought to be on a strict gluten free diet and the resulting difficulties to evaluate properly the impact of alternative therapies. They stress the need for complementary approaches to evaluate their pertinence and feasibility.

One important question remained as to whether the properties of the tested prolylendopeptidase were 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 CD patients. *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 µg/mL of peptide) and/or prolonged exposure were necessary to achieve complete digestion and prevent the intestinal transport of toxic or immunostimulatory fragments [18]. On the basis of *in vitro* digestion experiments or of perfusion experiments of rat small intestine, Koshla et al have suggested that, *in vivo*, PEP cooperates with BB enzymes to accelerate the breakdown of gliadin peptides [14]. We could not demonstrate a comparable effect when PEP was added onto the mucosal side of intestinal biopsies from patients with active CD mounted in Ussing chambers, a negative result likely due to the decreased activity of BB enzymes [13]. In contrast, we observed that the defect in intraluminal processing of prolin-rich peptides could be largely overcome, at least in controls and treated CD patients, 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 endosomo-lysosomal compartment of enterocytes. *Ex vivo* experiments with duodenal biopsies mounted in Ussing chambers showed that the 33 mer and 31-49 peptides, while resistant to proteolysis by BB 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 treated CD [13]. Not surprisingly, adding exogenous PEP into the mucosal compartment had no detectable impact on peptide transport and processing [18]. Incomplete luminal hydrolysis of proline-rich peptides may thus be compensated by epithelial processing in controls and treated CD patients. In treated CD patients, the tiny amount of peptides left undigested after transepithelial transport might however reach the threshold of immune reactivity [13]. In these patients, bacterial PEP, in spite of 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. Furthermore, 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 biopsies mounted in Ussing chambers suggested that oral therapy by PEP might be even “trickier” in patients with active CD. In the latter, in spite of the known alterations of tight junctions revealed by a decrease in electrical resistance of duodenal biopsies, there was no detectable paracellular leakage of peptides. Intestinal transport and processing of peptides were however profoundly altered [13]. Up to 50 % of the amount of peptide 31-49 or of the 33 mer placed on the mucosal surface were 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 CD, peptide transport seems modified from a non specific transcellular pathway to a “protected” transport allowing to escape from lysosomal enzymes ([13, 18] and Matysiak-Budnik et al, submitted). Thus, in active CD, oral therapy by bacterial PEP will have to overcome both the limited amount of BB enzymes due to villous atrophy and the protected transepithelial transport of gliadin peptides. Accordingly, it was necessary to add relative 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 on the capacity of PEP derived from *F. meningosepticum* to efficiently detoxify gluten in the duodenal lumen has very recently led two groups to seek new enzymes and/or investigate combination enzyme therapy. Koning and coworkers showed that a newly identified prolyl-endopeptidase produced by *Aspergillus niger*, not only hydrolysed gliadin peptides approximately 60 times faster than PEP from *F. meningosepticum* but was active within a much larger range of pH (2-8, optimum 4.5) . This enzyme could efficiently hydrolyse gluten in *in vitro* conditions mimicking the stomach and might therefore be useful to reduce markedly the amount of toxic peptides even before they enter the duodenum [19]. Koshla and coworkers have taken a slightly different but parallel approach. They first observed that recombinant EP-B2, a cysteine-protease derived from germinating barley seeds, is activated

at acid pH and by pepsin and can efficiently hydrolyse α2-gliadin *in vitro* in conditions mimicking the gastric lumen [20]. 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 [21]. Finally, these authors provided *in vitro* evidence that prior treatment by EP-B2 at low pH for one hour (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 [22].

In conclusion, despite some current drawbacks discussed above, oral therapy by proteases appears a promising simple approach that needs to be refined. First, the risk of side effects seems to be small. Short time *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 intact the bloodstream. 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 gastro-intestinal lumen and enhanced protection of the patients. Finally, even if oral protease therapy cannot fully replace the gluten-free diet, it might improve the quality of life of patients 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 from CD patients.

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Notes

The authors declare no competing interests

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