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Analytical Methods

Influence of dietary components on *Aspergillus niger* prolyl endoprotease mediated gluten degradation

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ABSTRACT

Celiac disease (CD) is caused by intolerance to gluten. Oral supplementation with enzymes like *Aspergillus niger* propyl-endoprotease (AN-PEP), which can hydrolyse gluten, has been proposed to prevent the harmful effects of ingestion of gluten. The influence of meal composition on AN-PEP activity was investigated using an *in vitro* model that simulates stomach-like conditions. AN-PEP optimal dosage was 20 proline protease units (PPU)/g gluten. The addition of a carbonated drink strongly enhanced AN-PEP activity because of its acidifying effect. While fat did not affect gluten degradation by AN-PEP, the presence of food proteins slowed down gluten detoxification. Moreover, raw gluten was degraded more efficiently by AN-PEP than baked gluten. We conclude that the meal composition influences the amount of AN-PEP needed for gluten elimination. Therefore, AN-PEP should not be used to replace a gluten free diet, but rather to support digestion of occasional and/or inadvertent gluten consumption.

1. Introduction

Celiac disease (CD) is a small intestinal disorder characterised by an abnormal immune response to gluten, a complex mixture of proteins with a high content of proline and glutamine residues. Given the inefficiency of gastric enzymes to cleave proline-rich protein sequences, gluten resists degradation (Hausch, Shan, Santiago, Gray, & Khosla, 2002; Kim, Quarsten, Bergseng, Khosla, & Sollid, 2004; Piper, Gray, & Khosla, 2004; Shan et al., 2002) and partly degraded gluten proteins reach the small intestine. Here they can be modified by the enzyme tissue tranglutaminase (TG2), which converts glutamine into the negatively charged glutamic acid. The introduction of one or more negative charges in these gluten peptides increases their binding to the disease predisposing HLA-DQ2 and HLA-DQ8 molecules. Pro-inflammatory $CD4^+$ T cells, specific for such HLA-DQ–gluten complexes, are typically found in patients with CD, but not in healthy controls, suggesting a crucial role in the disease process.

The only successful therapy for CD currently available is a strict lifelong gluten free diet (GFD), entailing the exclusion of all products containing wheat, barley and rye. Although a large number of *bone fide* gluten free food products are available, they tend to be less flavorful and more expensive than gluten-containing alternatives. Due to its favourable properties and low price, wheat gluten is widely used in the food industry. As a result, naturally gluten free foods may contain (traces of) gluten that was introduced deliberately or accidentally. In addition, gluten free products may incorporate gluten levels up to 20 ppm increasing the risk of inadvertent exposure of CD patients to gluten. Strikingly, about 50% of celiac patients following a GFD continue to suffer from symptoms and they still present with autoantibodies and/or villous atrophy (Lanzini et al., 2009), possibly due to inadvertent gluten exposure.

This situation highlights the need for additional measures to counteract the deleterious effects of gluten contaminants in the GFD. Oral enzyme supplementation is an attractive option due to its simplicity, ease and low risk of side effects. Oral lactase preparations have been used successfully by lactose intolerant patients (Shaukat et al., 2010). Likewise, oral enzyme supplementation to boost gluten degradation in the gastrointestinal tract has received much attention (reviewed in (Crespo Pérez, Castillejo de Villasante, Cano Ruiz, & León, 2012; Schuppan, Junker, & Barisani, 2009; Sollid

Abbreviations: AN-PEP, *Aspergillus niger* prolyl endoprotease; CD, celiac disease; TG2, tissue transglutaminase; GFD, gluten free diet; PEP, bacterial propyl oligopeptidase; PPU, proline protease unit; PVDF, polyvinylidene difluoride.

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& Khosla, 2011). Among these, oral administration of exogenous proline and glutamine specific proteases is one of the most investigated options (Mitea et al., 2007; Piper et al., 2004; Shan, Marti, Sollid, Gray, & Khosla, 2004; Siegel et al., 2006; Stepniak et al., 2006; Tye-Din et al., 2010).

Early investigations into oral protease therapy focused on bacterial prolyl oligopeptidase (PEP) (Hausch et al., 2002; Marti et al., 2005; Piper et al., 2004; Pyle et al., 2005; Shan et al., 2004, 2005). However, several studies conducted with PEP from *Flavobacterium meningosepticum* and *Myxococcus xanthus* revealed only moderate enzyme stability under simulated gastrointestinal conditions and low activity at stomach pH (Matysiak-Budnik et al., 2005; Shan et al., 2004; Stepniak et al., 2006). Although encapsulation has been proposed to protect them during stomach passage (Gass, Ehren, Strohmeier, Isaacs, & Khosla, 2005), it represents a sub-optimal solution. Ideally, the breakdown of gluten should take place in the stomach so that no gluten fragments reach the duodenum, the site where the immune response takes place.

In addition, a *Sphingomonas*-derived PEP was evaluated in a clinical trial in combination with other glutenases (Gass, Bethune, Siegel, Spencer, & Khosla, 2007; Tye-Din et al., 2010). Although these enzymes were found to reduce the gluten-specific T cell response, they did not significantly diminish CD-associated gastrointestinal symptoms (Tye-Din et al., 2010).

The use of PEP isolated from the food-grade fungus *Aspergillus niger* (AN-PEP) has also been proposed for gluten detoxification. Not only because this enzyme has clear advantages over bacterial PEP (Edens et al., 2005; Mitea et al., 2007; Stepniak et al., 2006) but also because it is available industrially. AN-PEP is optimally active at the low pH values typically found in the stomach and is resistant to degradation by pepsin. Further experiments have demonstrated its effective degradation of gluten proteins and peptides *in vitro* (Stepniak et al., 2006) and its efficient detoxification of gluten containing meals under *in vivo*-like conditions (Mitea et al., 2007). In this study, we elaborate on the optimal working conditions of AN-PEP under stomach-like conditions and report on how different dietary components impact AN-PEP activity.

2. Materials and methods

2.1. AN-PEP enzyme

The AN-PEP enzyme was obtained from DSM Food Specialties (Delft, The Netherlands). One Proline Protease Unit (PPU) is defined as the amount of enzyme that releases 1 μmol of p-nitroanilide per minute at 37 °C in a citrate/disodium phosphate buffer (pH 4.6) using 0.37 mmol/l Z-Gly-Pro-pNA (Bachem, Bubendorf, Switzerland) as substrate. The AN-PEP sample used has a specific activity of 15.7 PPU/g.

2.2. In vitro digestion

We performed all the *in vitro* digestions using the same procedure: a gluten mixture was prepared by mixing 2.2 g of wheat gluten powder (Amygluten 110, Syral, Aalst, Belgium, 77% protein of which 80% gluten) with artificial saliva (1 mmol/l $NaHCO_3$), and 18 μg/ml pepsin (Pepsin A, from Porcine Stomach Mucosa, ⩾250 units/mg, Sigma, USA) in a total volume of 275 ml and in the presence of indicated quantities of AN-PEP. To mimic stomach conditions, the gluten mixture was incubated under continuous stirring at 37 °C and the pH was reduced over time as indicated in the digestion scheme panel (Figs. 1A, 2A, 3A and 4A). Samples of 1 mL were collected at several time points and immediately stored at −80 °C until further analysis.

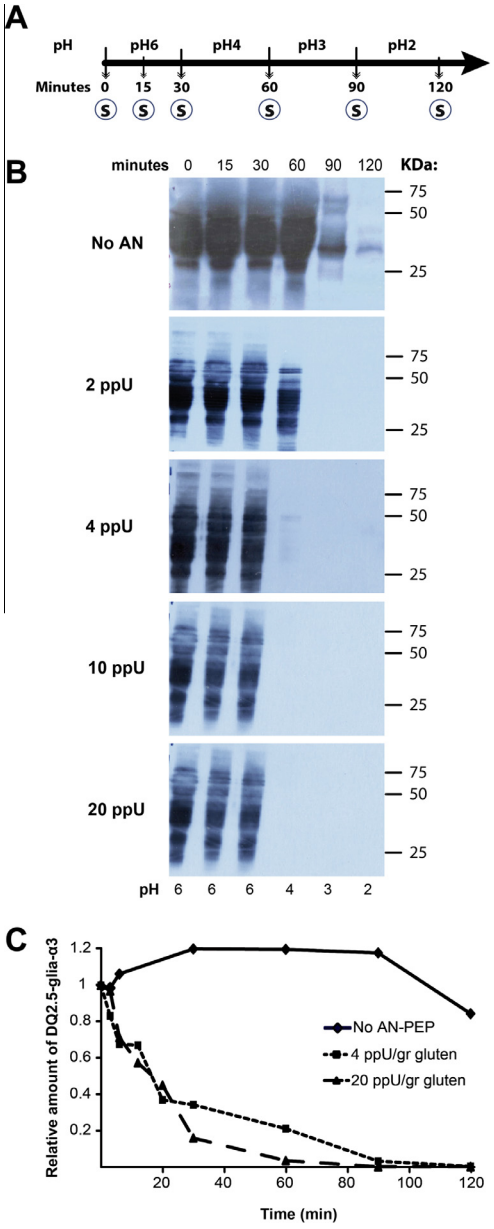

Fig. 1. AN-PEP degrades gluten powder in a dose-dependent manner. A suspension of gluten powder in water was incubated under stomach-like conditions in the presence of pepsin and various amounts of AN-PEP. The pH was lowered in time and samples were collected at the indicated time points (A). After enzyme inactivation, samples were separated into a water-insoluble and a water-soluble fraction. In the water-insoluble fractions the level of DQ2.5-glia-α1 epitopes was determined by Western blot (B), whereas in the water-soluble fractions the presence of DQ2.5-glia-α3 was determined by ELISA (C).

2.3. Testing of various parameters

To test the effect of different parameters minor modifications to the common procedure explained above were introduced in each case. The dose–response of AN-PEP, measured by gluten degradation, was determined by adding incremental amounts of enzyme to the gluten mixture. The effect of a carbonated drink was tested by adding 123 ml of a carbonated drink to the gluten mixture in a total volume of 275 ml. The effect of fat was tested in two sets of experiments. In the first one, 10 ml of sunflower or olive oil was added to the gluten mixture to a total volume of 275 ml (3.5% fat); in the second, 123 ml of skimmed milk (0.1% fat), or semi-skimmed milk (1.75% fat) or whole milk (3.5% fat) were added to the gluten