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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.

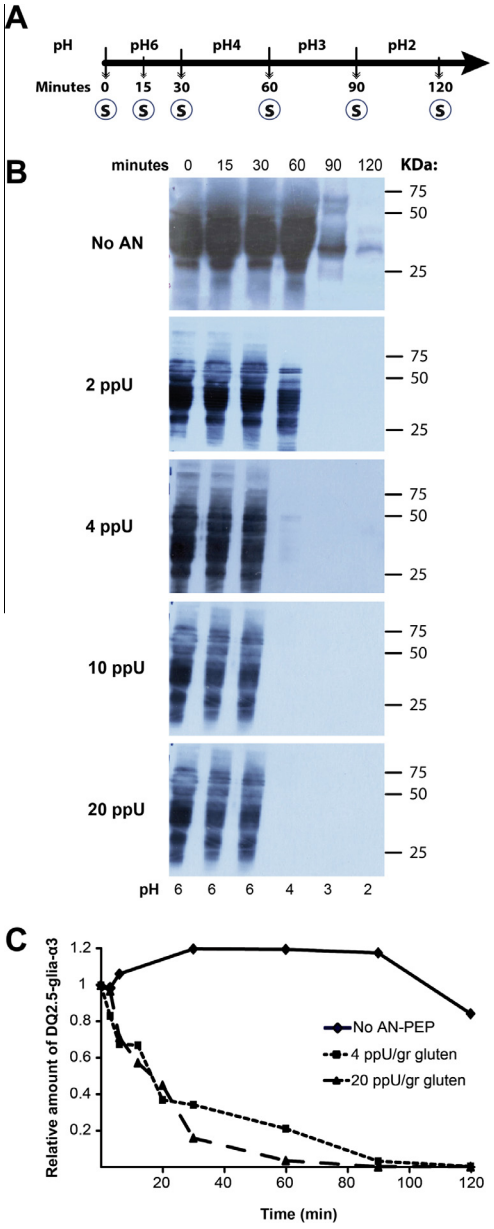

Fig. 1. AN-PEP degrades gluten powder in a dose-dependent manner. A suspension of gluten powder in water was incubated under stomach-like conditions in the presence of pepsin and various amounts of AN-PEP. The pH was lowered in time and samples were collected at the indicated time points (A). After enzyme 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

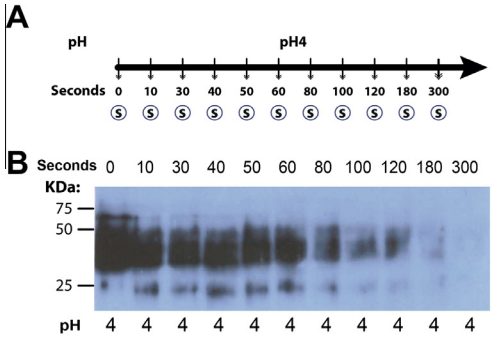

Fig. 2. AN-PEP kinetics at pH4. A suspension of gluten powder in water was incubated under stomach-like conditions at pH 4 in the presence of 20 PPU of AN-PEP/g gluten. The samples were taken at the indicated time points (A). From the water-insoluble fraction, the presence of DQ2.5-glia-α1 epitopes was determined by Western blot (B).

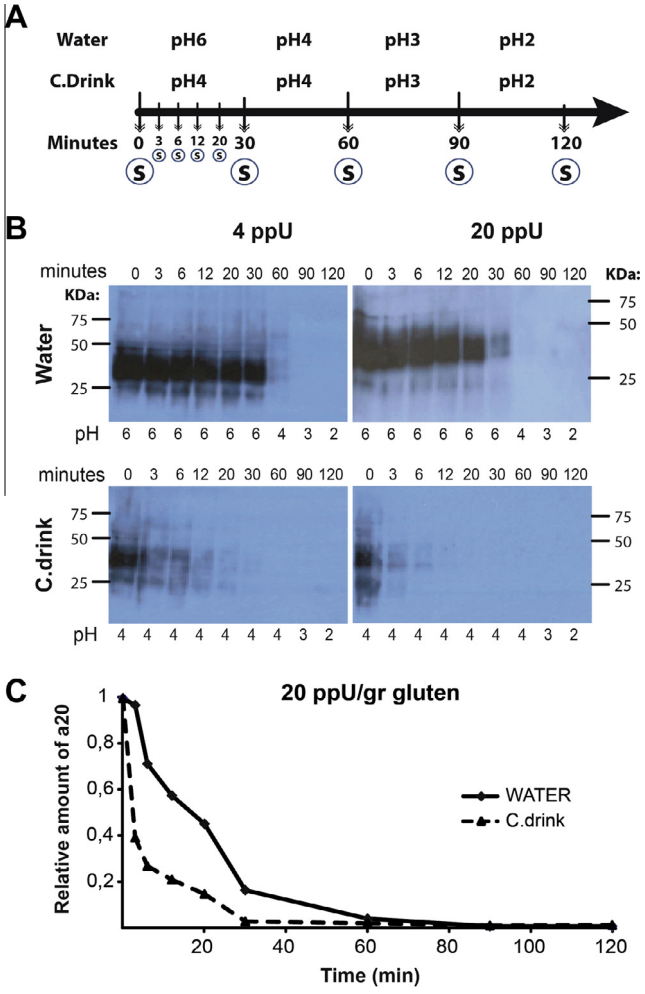

Fig. 3. AN-PEP activity in the presence of a carbonated drink. A suspension of gluten powder in water was incubated in the presence or absence of a carbonated drink under stomach-like conditions in the presence of either 4 or 20 PPU AN-PEP/g gluten. The samples were taken at indicated time points (A). From the water-insoluble fraction the presence of DQ2.5-glia-α1epitopes was determined by Western blot (B) and from the water-soluble fraction the presence of DQ2.5-glia-α3 was determined by ELISA (C).

mixture in a total volume of 275 ml. The role of supplementary dietary proteins was evaluated by adding either 4.3 g of whole egg powder (Whole egg powder, DSM, Delft, The Netherlands) or

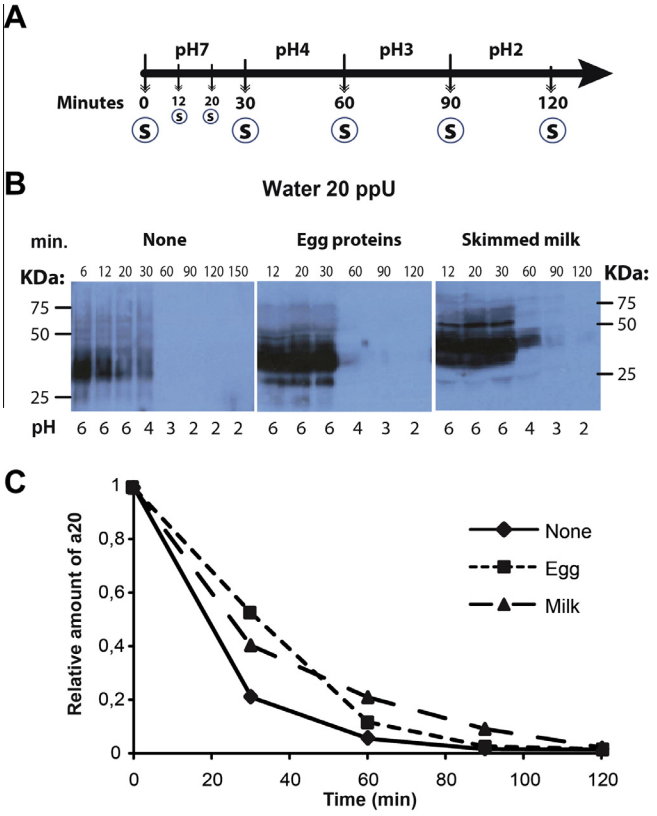

Fig. 4. AN-PEP activity in the presence of dietary proteins. Gluten powder in water was incubated with either whole egg powder or skim milk under stomach-like conditions in the presence or absence of 20 PPU of AN-PEP/g gluten. Samples were taken at the time points indicated (A). In the water-insoluble fractions the presence of DQ2.5-glia-α1 epitopes was determined by Western blot (B), while the presence of DQ2.5-glia-α3 in the water-soluble fractions was determined by ELISA (C).

123 ml of skimmed milk (containing 4.3 g of milk protein) to the gluten mixture. Gluten matrix effects were determined by comparing the degradation kinetics of gluten powder with an equivalent quantity of gluten present in toasted white wheat bread (Bolletje BV, Almelo, The Netherlands).

2.4. Sample pre-treatment

Before analysis, the samples collected during the *in vitro* gluten degradation experiments were thawed and AN-PEP residual activity was removed according to a previously established AN-PEP inactivation protocol (Mitea et al., 2007). Briefly, we increased the pH to 11–12 using 1 mol/l NaOH, followed by neutralisation with 1 mol/l HCl and incubation at 85 °C for 10 min. 1 ml from each sample was centrifuged for 10 min at 14,000 rpm to separate the water-soluble and insoluble fractions. Water-soluble fractions were kept at −80 °C until further analysis by ELISA. The water-insoluble fractions were dissolved in 100 µl of sample 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 and snap frozen at −80 °C until further analysis by Western blot.

2.5. Western blots

The level of T cell stimulatory epitopes in the water-insoluble fraction was determined by applying 25 µl of these fractions to 12.5% SDS–PAGE gel. After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes and

the proteins containing the DQ2.5-glia-α1 epitope were visualised using the monoclonal antibody 9.68 (Spaenij-Dekking, Kooy-Winkelaar, Nieuwenhuizen, Drijfhout, & Koning, 2004).

2.6. ELISA analysis

Water-soluble fractions were diluted 40–2000 times in PBS and the presence of the DQ2.5-glia-α3 epitope was quantified using the Gluten-Tec® ELISA assay (EuroProxima B.V., The Netherlands) according to the manufacturer's instructions.

3. Results

3.1. AN-PEP concentrations needed for gluten degradation

To determine the optimum concentration of AN-PEP needed to degrade gluten, the gluten mixture was incubated in the presence of four incremental doses of AN-PEP and gluten degradation was followed during a 2 h incubation period as depicted in Fig. 1A. The water-insoluble fractions were analysed by Western blot to determine the presence of the DQ2.5-glia-α1 epitope. In the water-soluble fractions, the presence of the DQ2.5-glia-α3 epitope was measured by a competitive ELISA (Gluten-Tec (Mujico et al., 2012)). 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 alpha-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 (Sollid, Qiao, Anderson, Gianfrani, & Koning, 2012).

The Western blot analysis indicated that in the absence of AN-PEP, degradation of the gluten proteins became apparent after 90 min, presumably due to pepsin activity. Advanced gluten degradation was observed after 120 min (Fig. 1B). Analysis of the soluble gluten fraction obtained under these conditions showed a similar slow gluten degradation pattern (Fig. 1C).

In the presence of a low concentration of AN-PEP (2 PPU/g gluten), degradation of the gluten proteins in the insoluble fraction became evident after 30 min, at pH 4, the optimum pH for AN-PEP activity (Fig. 1B) (Stepniak et al., 2006). In the presence of 4–20 PPU/g gluten, a more pronounced effect was observed and complete gluten degradation was observed by 60 min. The disappearance of the DQ2.5-glia-α3 epitope from the water-soluble fraction corroborates this observation (Fig. 1C). Notably, gluten peptides were significantly reduced even before the optimal pH for AN-PEP activity was reached. The latter observation is in agreement with previous reports showing that AN-PEP is active in the pH range 2–8, with an optimum at pH 4 (Stepniak et al., 2006).

Thus, gluten degradation under simulated gastric conditions was enhanced by the presence of AN-PEP, with the strongest effect observed at a concentration of 20 PPU/g gluten.

3.2. Kinetics of gluten degradation by AN-PEP at pH 4

As our data demonstrated AN-PEP was particularly effective at pH 4, we determined the kinetics of AN-PEP degradation at pH 4. For this purpose the gluten mixture was incubated with 20 PPU AN-PEP/g gluten (at pH 4) under stomach-like conditions. During a brief 5 min incubation, multiple samples were collected (Fig. 2A) and each sample was analysed by Western blot for the presence of gluten. Our results demonstrated that 5 min was sufficient to degrade the gluten such that it can no longer be detected by Western blot analysis (Fig. 2B).

3.3. A carbonated drink improves AN-PEP mediated gluten degradation

Since low pH conditions accelerated the AN-PEP mediated gluten degradation, we hypothesised that the presence of acidic food components might have a similar effect. Taking into account the popularity and low pH of carbonated drinks, we determined the rate of AN-PEP mediated gluten degradation in the presence of a carbonated drink. For this purpose, a gluten mixture was prepared with a total volume of 275 ml of which 123 ml was a carbonated drink, and gluten degradation was determined in the absence or presence of either 4 or 20 ppU/g of gluten. Samples were taken at various time points according to the scheme depicted in Fig. 3A. The presence of gluten proteins and peptides was determined by Western blot (Fig. 3B) and by ELISA (Fig. 3C). The addition of the carbonated drink was found to lower the pH of the starting mixture from 6 to 4 (Fig. 3A), and this resulted in significantly faster degradation of gluten, particularly at the highest enzyme concentration.

3.4. Supplementary dietary proteins affect AN-PEP mediated gluten degradation

A meal usually contains plant as well as animal proteins, which are a potential substrate for AN-PEP and may thus slow down gluten degradation. As AN-PEP is highly specific for proline residues (Edens et al., 2005), we selected two sources of animal protein with different proline contents (whole egg proteins [4% proline] and milk proteins [10% proline]) and determined the impact on AN-PEP mediated gluten degradation according to the scheme depicted in Fig. 4A. While in the absence of additional proteins gluten was fully degraded within 60 min (Fig. 4B), in the presence of additional proteins, milk proteins in particular, gluten degradation was slower (Fig. 4B and C).

As our previous experiments had shown that the presence of a carbonated drink improved gluten degradation by AN-PEP, we tested whether the addition of a carbonated drink would counteract the negative effects of additional animal proteins. Indeed, the addition of the carbonated drink partially compensated for the presence of additional food proteins (not shown).

3.5. Effect of fat on AN-PEP activity

In addition to proteins, dietary fat might also interfere with gluten detoxification by AN-PEP. To test for any inhibitory or stimulatory effects, we tested the impact of a variety of fat sources on AN-PEP activity in two sets of experiments. In the first, we determined the impact of skimmed milk (0.1% fat), semi-skimmed milk (1.7% fat), and whole milk (3.5% fat). In a second, we tested the effect of sunflower oil and olive oil (3.5% fat). The results indicate that neither of the fat sources had any significant effect on the rate of gluten degradation by AN-PEP (Fig. 5A–C).

3.6. Effect of food matrix on AN-PEP gluten degradation

The gluten fraction in food is typically incorporated in a matrix structure that endows the product with its textural characteristics. To determine the impact of such a matrix on gluten accessibility, we compared the degradation kinetics of raw gluten powder with gluten present in toast following the scheme depicted in the Fig. 1A. Degradation of gluten was determined by Western blot analysis. Complete degradation of the gluten in the toast required an additional 60 min compared with gluten powder under the same conditions (Figs. 6 and 1B).

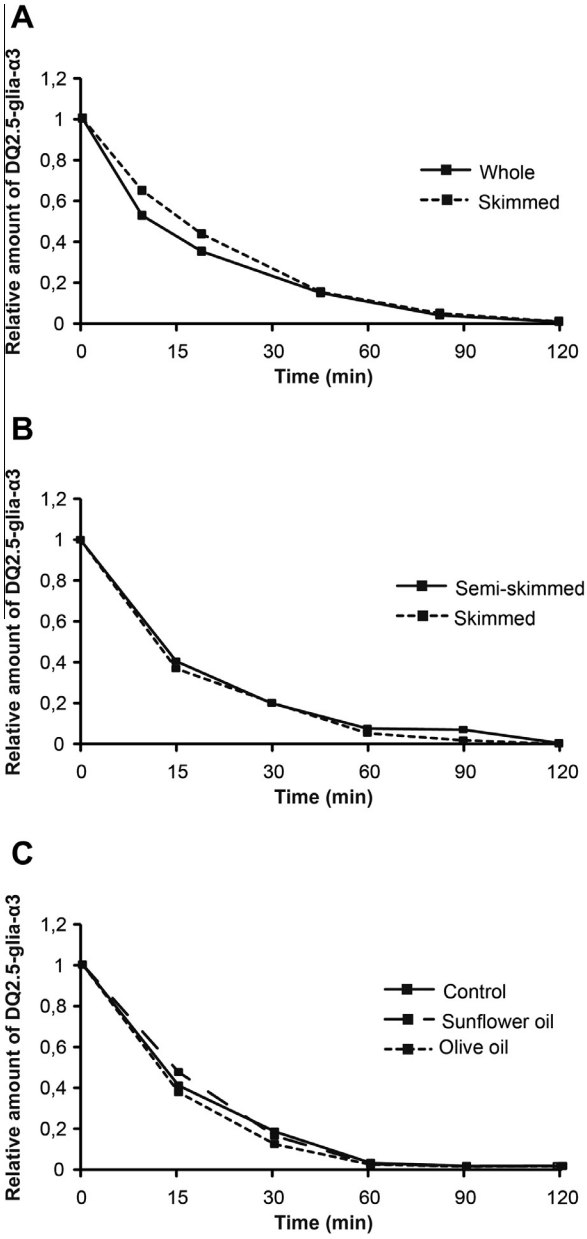

Fig. 5. AN-PEP activity in the presence of fat. Gluten powder was suspended in either a 1:1 mix of water/skim milk or water/whole milk and incubated under stomach-like conditions in the presence of 20 ppU of AN-PEP/g gluten. The presence of DQ2.5-glia-α3 epitopes in the water-soluble fractions of samples taken at the time points was determined by ELISA.

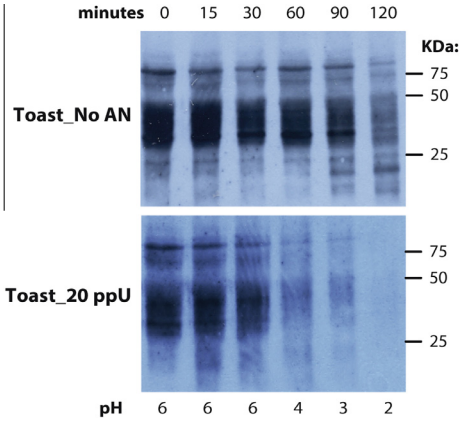

Fig. 6. AN-PEP efficacy depends on food matrix. Gluten powder or a slice of crushed white bread were suspended in water and incubated under stomach-like conditions in the absence or presence of 20 PPU of AN-PEP/g gluten. The presence of DQ2.5-glia-α1 epitopes in the water-insoluble fractions of samples taken at the time points was determined by Western blot.

4. Discussion

In this study, we demonstrated that AN-PEP efficiently hydrolysed gluten, particularly at pH 4, and such conditions could be achieved by mixing the gluten with a carbonated drink.

Two commonly consumed fat sources, namely milk and plant oils, containing different ratios of saturated versus unsaturated fatty acids (milk: 2 to 1; olive oil: 1 to 6; sunflower oil: 1 to 8), had no impact on AN-PEP activity under the experimental conditions used. This is of particular interest in view of *in vivo* observations that fatty, high caloric meals are known to result in longer stomach transit times (Kwiatek et al., 2009), which would facilitate complete gluten degradation by AN-PEP during stomach passage. In contrast, the addition of proteins did have a negative effect on AN-PEP-mediated gluten degradation. In particular, milk proteins had the most harmful effect, presumably because of the high proline content of casein, the most abundant protein in milk.

Finally, we demonstrated that gluten present in toast was degraded more slowly than raw gluten. It should be noted, however, that no chewing was simulated prior to exposure to AN-PEP. *In vivo*, amylase and other enzymes in saliva initiate food degradation during chewing, which may have a positive effect on the gluten degradation by AN-PEP in the stomach.

We previously demonstrated that AN-PEP is fully active under stomach-like conditions and not degraded by pepsin (Mitea et al., 2007; Stepniak et al., 2006). In this study, we confirmed these observations and further demonstrated that the amount of AN-PEP needed for complete gluten elimination is influenced by the composition of the meal. The results obtained in this study will help in the preparation of guidelines for the use of AN-PEP as an oral supplement for gluten intolerant patients. Importantly, the fact that we have demonstrated that the addition of a carbonated drink increases the efficiency of gluten degradation by AN-PEP suggests that any dietary component that reduces chyme pH could improve AN-PEP activity and, thus, reduce gluten detoxification time. In fact, the negative effects of dietary proteins on AN-PEP activity were counteracted with the addition of a carbonated drink.

In conclusion, optimal conditions for AN-PEP mediated gluten degradation can be defined. However, since the amount of AN-PEP needed for complete gluten elimination is influenced by the composition of the meal, oral supplementation with AN-PEP should not be used as a replacement to GFD, but rather to support the digestion of incidental and/or inadvertant gluten consumption.

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Conflict of interest

Luppo Edens and Maaike J. Bruins are employees of DSM Food Specialties and co-inventors of a number of patents claiming the use of AN-PEP as possible tool for destroying toxic gluten epitopes.

The authors declare no competing financial interest.

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