



Treatment of both native and deamidated gluten peptides with an endo-peptidase from *Aspergillus niger* prevents stimulation of gut-derived gluten-reactive T cells from either children or adults with celiac disease

Henrik Toft-Hansen^{a,b,*}, Karina S. Rasmussen^{a,b},
Anne Staal^{a,b}, Erwin L. Roggen^c, Ludvig M. Sollid^d, Søren T. Lillevang^b,
Torben Barington^b, Steffen Husby^a

^a Hans Christian Andersen Children's Hospital, Odense University Hospital, University of Southern Denmark, Denmark

^b Department of Clinical Immunology, Odense University Hospital, University of Southern Denmark, Denmark

^c Immunotoxicology, Novozymes, Bagsværd, Denmark

^d Centre for Immune Regulation, University of Oslo, Norway

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Abstract Celiac disease (CD) is characterized by an inappropriate immunological reaction against gluten driven by gluten-specific CD4+ T cells. We screened 25 proteases and tested 10 for their potential to degrade gluten *in vitro*. Five proteases were further tested for their ability to prevent the proliferative response by a gluten-specific CD4+ T cell clone and seven gluten-reactive T cell lines to protease-digested gluten peptides. A proline-specific endo-peptidase from *Aspergillus niger* (AnP2) was particularly efficient at diminishing proliferation after stimulation with cleaved antigen, and could completely block the response against both native and deamidated gluten peptides. We found that AnP2 was efficient down to a 1:64 protease:substrate ratio (w:w). When AnP2 was tested in assays using seven gluten-reactive T cell lines from individual CD patients (three adults and four children), the response to gluten was diminished in all cases. Our study indicates a therapeutic benefit of AnP2 to CD patients.

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* Corresponding author at: Hans Christian Andersen Children's Hospital, Pediatric Research Unit, Odense University Hospital, Sdr. Boulevard 29, 5000 Odense C, Denmark. Fax: +45 65911862.

E-mail address: htoft-hansen@health.sdu.dk (H. Toft-Hansen).

1. Introduction

Celiac disease (CD) affects about 1% of the population in western countries and appears to be increasing in prevalence [7,8,16,21]. Ingestion of gluten causes an inappropriate immunological reaction in CD patients driven by gluten-specific CD4⁺ T cells in individuals with tissue type HLA DQ2 or DQ8 [29]. Gluten and related prolamins are present in wheat, rye and barley, and the immune response in adults with CD is directed against epitopes from all three types of cereal [38]. CD requires a combination of genetic and environmental factors to develop [1], but the exact cause of the disease is unknown, and there is no treatment other than a life-long gluten-free diet. The normal response to food proteins such as gluten is a state of immunological tolerance maintained by the mucosal immune system [41]. These tolerance-inducing mechanisms are for unknown reasons insufficient in CD patients, in whom ingestion of gluten causes intestinal inflammation, particularly in the upper part of the small intestine, leading to malabsorption, and a wide range of both intestinal and systemic symptoms [6].

The ubiquitous enzyme transglutaminase 2 (TG2) is pathogenic in CD by deamidating (changing some of the glutamine amino acid residues (Q) to glutamic acid (E) residues) gluten peptides, thereby making them better suited for antigen presentation on HLA DQ2 or DQ8 [19]. Occurrence of anti-TG2 auto-antibodies correlates with the intake of gluten and the severity of intestinal damage in CD patients [5]. This correlation between presence of an autoantibody and disease symptoms illustrates a likely autoimmune element in the pathogenesis [30].

In other clinical conditions, enzyme preparations have been used successfully as a food additive such as lactase for lactose intolerance [14] and pancreatic enzyme replacement therapy of cystic fibrosis patients with pancreatic insufficiency [15]. A similar approach has been suggested as a therapeutic avenue in CD as reviewed in [3,13,31,33]. Gluten-derived peptides are not cleaved very efficiently by digestive proteases in the human gastrointestinal tract, probably due to the high proline-content of about 15% in gluten [35], since proline protects against proteolysis [11]. Relatively large peptides have been demonstrated to be highly immunogenic in CD, such as the 33-mer from alpha-gliadin containing three overlapping epitopes [25].

A potential therapeutic strategy for CD patients could be to use a protease as food supplement to degrade immunogenic peptides from gluten in the digestive system, before an inappropriate immune reaction is initiated. We tested five proteases *in vitro* for their potential to prevent the proliferative response to protease-digested gluten substrates by a gluten-specific CD4⁺ T cell clone as well as seven gluten-reactive T cell lines from individual CD patients (three adults and four children).

2. Materials and methods

2.1. SDS-PAGE

Protein fragmentation was visualized by protein gel electrophoresis with NuPage™ Bis-Tris gels as prescribed by the manufacturer (Invitrogen, Carlsbad, CA, USA). Ultra Low

Range (20×) (Sigma-Aldrich, Brøndby, Denmark) and Mark 12™ Wide Range Protein Standard (Invitrogen) were used as molecular weight markers diluted 20× in 1:1 Tris-Tricine sample buffer/water (1:1). Five microliters was loaded on the gel. Visualization of the protein fragments was performed using the ProteoSilver Plus Silver Staining Kit (PROT-SIL2; Sigma-Aldrich) as recommended by the manufacturer.

2.2. Cells

The gluten-reactive T cell clone TCC535.3.23 was generated from a duodenal biopsy from an adult CD patient at Oslo University Hospital according to published methods [20]. TCC535.3.23 is specific for the deamidated peptide sequence QLQPFQPELPY derived from the highly immunogenic alpha-gliadin 33-mer [25]. Duodenal biopsy-derived T cell lines (TCLs) from children and adults with CD were generated as described [20], either at Oslo University Hospital or Odense University Hospital.

2.3. Preparation of substrates

Preparations of 100 mg of gluten or gliadin (Sigma-Aldrich) were suspended in 10 ml 10 mM acetic acid, and sonicated for 60 min at room temperature. The resulting suspension was filtered through a 40 µm filter to remove insoluble parts. The protein suspension was solubilized with chymotrypsin (CT) (Sigma-Aldrich) according to the manufacturer's protocol, followed by heat-inactivation of CT at 98 °C for 10 min, and the final protein concentration was determined. For deamidation, the substrates were incubated with guinea pig transglutaminase (TG) (Sigma-Aldrich) 1:20 (w:w) at 37 °C for 2 h, followed by heat-inactivation of TG for 10 min at 98 °C. The 33-mer peptide was synthesized in the deamidated version (LQLQPFQPELPYPQPELPYPQPELPYPQPF) (Genscript, Piscataway, NJ, USA) and reconstituted in water.

2.4. Protease treatment

Proteases were supplied by Novozymes, Bagsværd, Denmark. Antigen substrates were diluted in buffer (100 mM Tris-HCl, 10 mM CaCl₂, pH 4–5) to a concentration of 200 µg/ml and treated with proteases diluted in the same buffer at different protease to substrate ratios. Protease treatment was carried out at pH 4–5 for 2 h at 37 °C, either before or after deamidation of substrates with TG. Sham treatments were performed by heat-inactivating the protease preparation for 10 min at 98 °C prior to addition to substrates.

2.5. T cell proliferation assays

T cell proliferation assays were performed using deamidated or non-deamidated antigens, with irradiated HLA DQ2 homozygous B cells as antigen presenting cells (VAVY cell line from International Histocompatibility Working Group, Seattle, WA, USA). A suspension of 75 µl of B cells (1 million/ml) was incubated with 25 µl of antigen (final concentration 25 µg/ml) on day zero, and 50 µl of T cells (0.5–1 million/ml) added on day one. Proliferation was measured on day four by incorporation of radioactively labeled thymidine

added 16–18 h prior to harvest of cells. The extent of proliferation was calculated as a stimulation index (SI), where the value in counts per minute for the sample was divided by the mean value of the negative control (B cells incubated with T cells without presence of antigen).

2.6. Statistical analysis

One-way ANOVAs with either Dunnett's or Bonferroni's multiple comparison tests were applied to analyze results using the GraphPad Prism software (GraphPad Software Inc., San Diego, California, USA), as indicated in figure legends. A statistical significance limit of $p < 0.05$ was chosen. Error bars represent standard deviations.

3. Results

3.1. Selection of proteases

From a list of 25 proteases representing various families, 10 enzymes were selected on the basis of their pH activity range, pH optimum and pH stability (Table 1). The number of enzymes was further reduced from 10 to five enzymes after *in vitro* assessment of their activity on gluten substrate in protease:substrate ratios ranging from 1:1000 to 1:1 million. The proteolytic activity on these substrates after 30, 45 and 60 min of incubation at 37 °C was visualized by SDS–PAGE and silver staining. Among the ten proteases, only AnP2 showed consistent degradation of gluten substrate (Fig. 1A and B). The other nine proteases did not show significant degradation at a ratio of 1:1000 exemplified by P1 in Fig. 1C.

3.2. Treatment of native (non-deamidated) gluten substrates with *Aspergillus niger* protease 2 completely prevents response

We tested the efficacy of proteases P1, AnP2 and P3 in terms of preventing proliferation of TCC 535.3.23 after protease-treatment of native (chymotrypsin (CT)-treated) gluten (Fig. 2). Gluten was treated with proteases for 2 h at 37 °C at a 1:1 protease:substrate ratio, followed by heat-inactivation of proteases, and subsequent deamidation. This would mimic

the situation, where the proteases act on gluten antigen substrates before these are deamidated in the GI tract. Compared to sham-treated controls, P1 and P3 had either no or moderate effect, whereas AnP2 completely abolished reactivity to a level equivalent to the negative control where no antigen was added. AnP2 also completely abolished reactivity to the 33-mer, which was synthesized in the deamidated form and protease-treated in the same way as CT-gluten. The effect of AnP2 was equally convincing using native gliadin as substrate (not shown).

3.3. Treatment of deamidated gluten substrate with *Aspergillus niger* protease 2 completely prevents response

We then tested whether the three selected proteases (AnP2, P1 and P3) could abolish reactivity by cleaving deamidated gluten and gliadin substrates in a protease:substrate ratio of 1:1. This would mimic the situation, where proteases act on gluten antigen substrates after the antigens have been deamidated in the gastrointestinal (GI) tract. Chymotrypsin-treated deamidated antigen substrates were treated with proteases for 2 h at 37 °C followed by heat-inactivation of proteases. We found equivalent results to the experiment, where native gluten substrate was used (Fig. 2), i.e. that AnP2 abolished reactivity to a level equivalent to the negative control, whereas P1 and P3 had either no statistically significant effect or only a moderate effect on proliferation in response to gluten or gliadin compared to sham-treated controls. In a separate experiment, treatment with P4 and P5 had no statistically significant effect on the response, whereas AnP2 again completely abolished reactivity (data not shown). Given that we found equivalent results of protease activity using either native or deamidated substrates, we have used only deamidated substrates in subsequent experiments.

3.4. *Aspergillus niger* protease 2 is efficient down to a 1:64 ratio of protease to substrate

Given the unpractically high protease:substrate ratio (1:1) used in experiments for Fig. 2, we aimed to titrate the amount of AnP2 needed to abolish proliferation (Fig. 3A). We

Table 1 List of proteases investigated.

Protease name	Type	Donor	Family	pH range	pH optimum	pH stability	Other characteristics
P1	Endo-peptidase	<i>Thermoascus aurantiacus</i>	M35	4–10	6	3.5–10.5	
P2 (AnP2)	Endo-peptidase	<i>Aspergillus niger</i>	S28	3.5–5	4	2.5–7.5	Pro (P1 position)
P3	Endo-peptidase	<i>Bacillus licheniformis</i>	S1B	7–10	9	4.5–10	P1: Glu
P4	Endo-peptidase	<i>Zophobas atratus</i>	C1A	3.5–6	5	4.5–6.5	
P5	Endo-peptidase	<i>Alicyclobacillus</i> sp.	G1	<2–6.5	3	<2–9.5	
P6	Endo-peptidase	<i>Aspergillus oryzae</i>	S53	3–5	4	3.5–6	P1: Lys or Arg
P7	Endo-peptidase	<i>Bacillus amyloliquefaciens</i>	M4	5.5–8.5	9	5–10	P1': hydrophobic aa
P8	Endo-peptidase	<i>Rhizomucor miehei</i>	A1	2–4.5	4	3–9	
P9	Endo-peptidase	<i>Aspergillus oryzae</i>	A1	3–5.5	4	3.5–5.5	
P10	Oligo-peptidase	<i>Spingomonas capsulata</i>	S9A	6–10	8	5–9	

Ten enzymes were selected on the basis of their pH activity range, pH optimum and pH stability as potential gluten-degrading enzymes. After *in vitro* assessment of their activity on gluten and gliadin substrates, the number of enzymes chosen for further analysis was reduced from ten to five enzymes (P1–5).

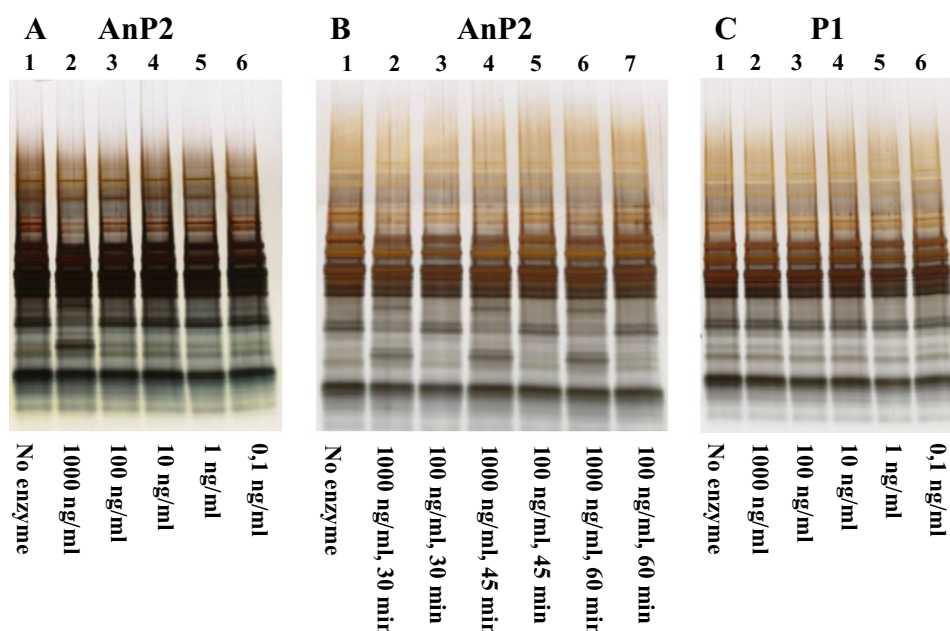


Figure 1 Gluten degradation. Gluten degradation was evaluated with SDS–PAGE. Native gluten was incubated at 37 °C with AnP2 enzyme at various concentrations for 30 min (A) or longer (B); or with P1 at various concentrations for 30 min (C). Panels A and C: lanes: 1: gluten alone; 2–6: 1 mg/ml gluten incubated with AnP2 in decreasing concentrations. Panel B: lanes: 1: gluten alone; 2–7: 1 mg/ml gluten incubated with AnP2 at either 1000 or 100 ng/ml for 30, 45 or 60 min.

saw a complete inhibition of proliferation after protease treatment down to a protease:substrate ratio of 1:64. A ratio of 1:50 was chosen for the following experiments.

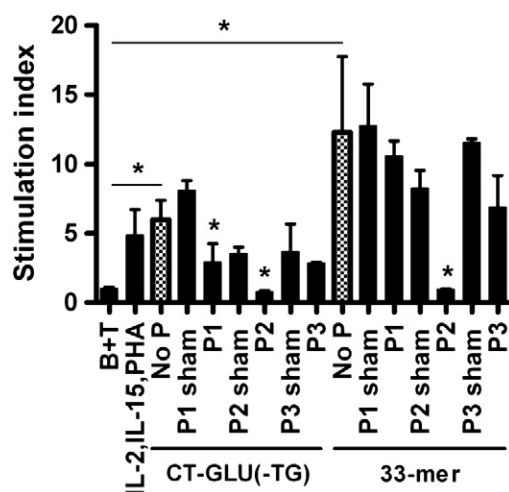


Figure 2 Protease-treatment of native gluten antigen substrate. Proliferation of the gluten-reactive TCC 535.3.23 after stimulation with native chymotrypsin-treated gluten which was deamidated after protease-treatment. For each antigen separately, the unstimulated B + T control was compared to the non-protease-treated control (shaded columns) and each protease-treatment was compared to the corresponding sham-treated control in a one-way ANOVA followed by Bonferroni's multiple comparison post test. Sham-treatment controls were treated with heat-inactivated protease. The mean value of unstimulated control samples (B + T) is set to 1 and other values are expressed as relative values (stimulation indices). CT-GLU(-TG): chymotrypsin-treated gluten deamidated after protease treatment * $p < 0.05$, $n = 3$ in each group.

3.5. The gluten-reactive CD4+ T cell clone reacts against deamidated gluten antigens only

To confirm the specificity of the T cell clone (TCC) 535.3.23, we tested it in proliferation assays with CT-treated gliadin or gluten in native or deamidated form, as well as with the 33-mer as positive control (Fig. 3A and B). TCC 535.3.23 was previously shown to proliferate in response to stimulation with the 33-mer epitope QLQPFQPQLPY (QLQPFQPQLPY in the native form) (data not shown). Native (CT-treated, but not deamidated) gluten did not induce proliferation illustrating the need for deamidation (Q to E in position 9) for epitope recognition by this T cell clone.

3.6. The response to gluten by seven gluten-reactive T cell lines is diminished by AnP2-treatment of substrates

To verify the efficacy of the protease treatment in each round of experiments, a control experiment using the clone TCC 535.3.23 previously shown to respond virtually 100% to AnP2-treatment of substrates, was included in each round of protease treatment. A representative result is shown in Fig. 3B. All seven TCLs tested (three from adults (Fig. 4A–C) and four from children (Fig. 4D–E) showed a response to AnP2 treatment evident by a statistically significant decrease in proliferation compared to sham-treated controls after stimulation with both gluten and the 33-mer (Fig. 4A–G).

4. Discussion

In this study we selected 10 proteases from a group of 25 based on their pH activity range, pH optimum and pH

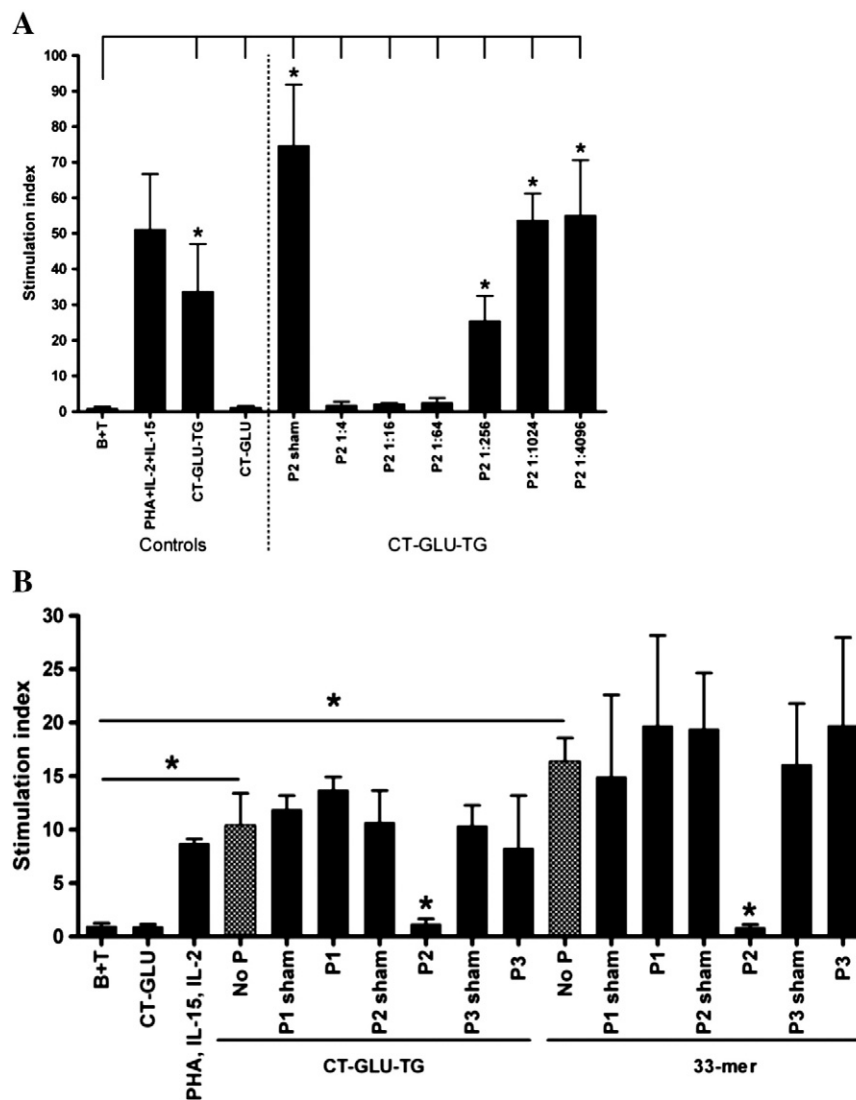


Figure 3 Proliferation of TCC 535.3.23, and titration of the AnP2 protease:substrate ratio. Panel A: titration of AnP2 incubated at various ratios with 25 µg/ml deamidated gluten. All samples except the positive control stimulated with PHA, IL-15 and IL-2 were compared to the unstimulated control (B + T) in a one-way ANOVA followed by Dunnett's multiple comparison post test. Panel B: proliferation of the T cell clone 535.3.23 in response to deamidated or native gluten, and after protease-treatment of antigen substrates. For each antigen separately, the unstimulated B + T control was compared to the non-protease-treated control in a one-way ANOVA followed by Bonferroni's multiple comparison post test. The B + T control was also compared to stimulation with native CT-GLU. Sham-treatment controls were treated with heat-inactivated protease. The mean value of unstimulated control samples (B + T) is set at 1 and other values are expressed as relative values (stimulation indices). CT-GLU: chymotrypsin-treated gluten (native); CT-GLU-TG: deamidated chymotrypsin-treated gluten; * $p < 0.05$; $n = 3-8$ in each group (A), and $n = 3-6$ in each group (B).

stability considering the physiological conditions in the upper gastrointestinal tract, and reduced further to five proteases after *in vitro* assessment of their activity on gluten and gliadin substrates at a ratio of 1:1000. At this ratio, only AnP2 showed convincing gluten degradation. The five proteases were tested in proliferation assays on their ability to prevent stimulation of gluten-reactive T cells after pretreatment of gluten antigens. Four of the proteases showed moderate or no effect, but treatment with AnP2 of both native (non-deamidated) and deamidated gluten substrates completely prevented response by a gluten-reactive T cell clone with specificity for an epitope within the 33-mer

peptide at ratios of protease to substrate down to 1:64 (w:w). When seven gluten-reactive T cell lines from individual CD patients, which represent a broader range of epitope specificities, were used, the effect of AnP2 treatment was evident in experiments with all seven T cell lines (from three adults and four children).

Staining of TG2 reveals presence of the enzyme in the extracellular matrix of the lamina propria as well as in the luminal brush border [29] although it is still unclear where active enzyme does turnover gluten peptides as most enzyme appears to be inactive *in vivo* [28]. Therefore, we found it relevant to test efficacy of protease-treatment both

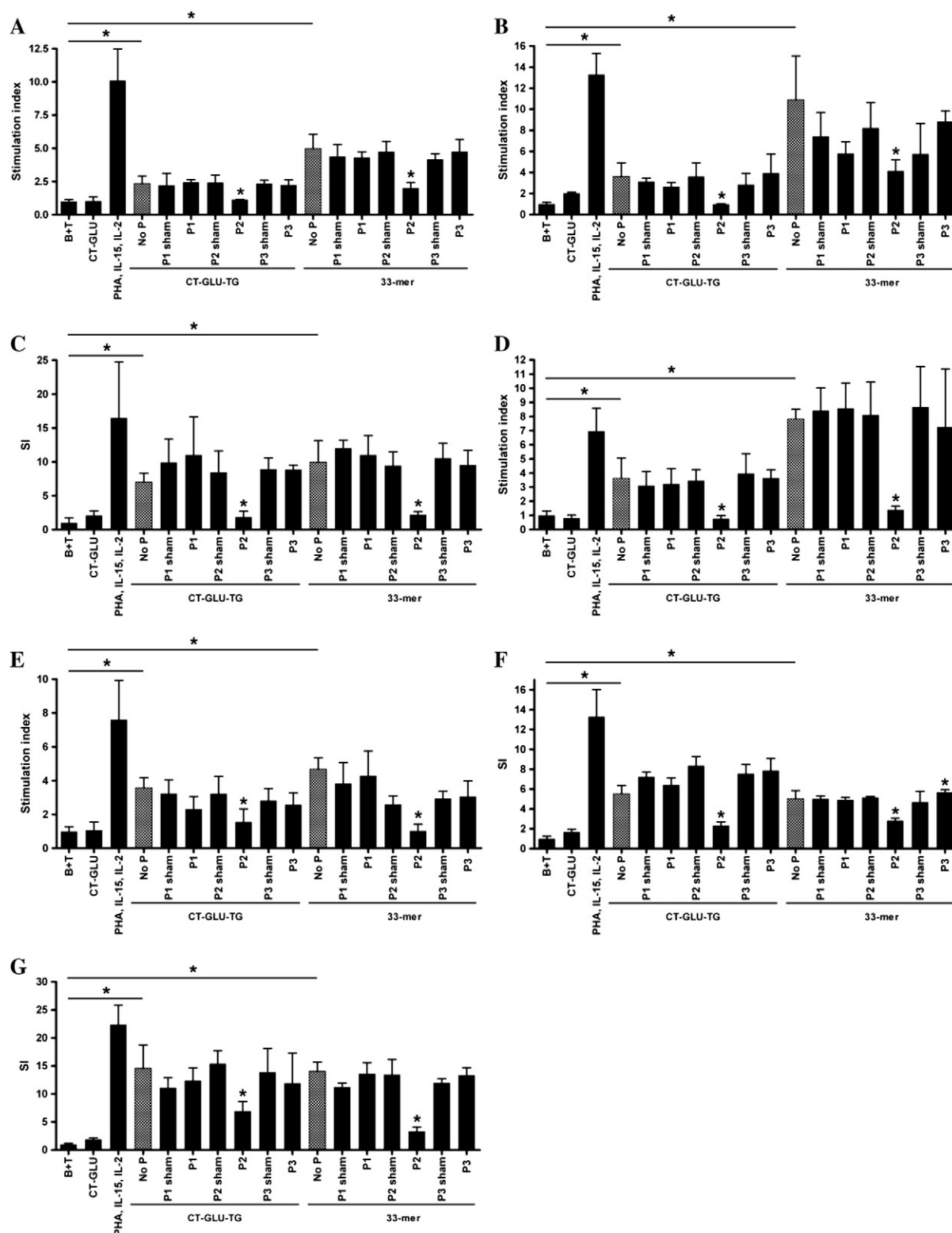


Figure 4 Proliferation of seven T cell lines. Panels A–G: proliferation of the T cell lines (A–C from adult patients, D–G from children) in response to deamidated or native gluten, and after protease-treatment of antigen substrates. T cell lines used: Panel A: A535-3; Panel B: A678-1; Panel C: Dv13-3; Panel D: B3-3; Panel E: C1-1; Panel F: B7-1; Panel G: D12-3. For each antigen separately, the unstimulated B + T control was compared to the non-protease-treated control (shaded columns) and each protease-treatment was compared to the corresponding sham-treated control in a one-way ANOVA followed by Bonferroni's multiple comparison post test. Sham-treatment controls were treated with heat-inactivated protease. The mean value of unstimulated control samples (T + B) is set to 1 and other values are expressed as relative values (stimulation indices). CT-GLU: chymotrypsin-treated gluten (native); CT-GLU-TG: deamidated chymotrypsin-treated gluten; * $p < 0.05$; $n = 3$ –6 in each group.

before and after deamidation of substrates. Both versions of gluten peptides could be present in the stomach and lumen of the duodenum, where AnP2 should exert the proteolytic effect on gluten before immunogenic gluten peptides reach the lamina propria of the small intestine. AnP2 worked equally well in our experiments for both native and deamidated gluten substrates.

Our results from assays using a T cell clone are very consistent, whereas the results are more variable when using T cell lines. These data probably relate to the higher heterogeneity and multiple T cell specificities of the TCLs, whereas the TCC consists of mono-specific CD4+ T cells. However, it may be more physiologically relevant to use TCLs, being more representative of the actual immune response in a CD patient. It has been reported that T cell responses in children may show different reactivities than the responses observed in adult CD patients [39]. In our study, we used seven T cell lines derived from seven different CD patients, both adults and children, and showed a convincing effect of AnP2 treatment in all seven. AnP2 treatment was efficient in assays using both TCLs from children as well as adults.

AnP2 is a proline-specific endo-peptidase from *Aspergillus niger*. Our results are consistent with previously published results by other groups using a prolyl endoprotease from *Aspergillus niger* (AN-PEP) [18,34] where it was shown that AN-PEP could prevent stimulation of gluten-reactive T cell clones. We also demonstrated that a protease from *Aspergillus niger* is extremely efficient at degrading gluten *in vitro*, and showed that AnP2 can reduce a response by a gluten-reactive T cell clone. We furthermore demonstrated that AnP2 can reduce the response by gluten-reactive T cell lines, which contain T cells presumably with multiple specificities for gluten epitopes. In our study we included a titration assay to test if AnP2 would be active at a protease:substrate ratio relevant for clinical application. We found that AnP2 was effective down to a 1:64 (w:w) ratio. Given that a normal western diet contains approximately 13 g of gluten per day [40], the ratio of 1:64 translates to an amount of protease of approximately 200 mg per day needed to cleave all the gluten ingested, if bioavailability and proteolysis is 100% efficient, which, however, is unlikely to be the case. As predicted, our results show increased gluten antigen degradation with increased incubation time and protease:substrate ratio. If these factors are not optimal in a clinical setting, and gluten degradation is incomplete, there is a risk that the remaining gluten antigens could provoke symptoms. However, a protease food additive could help bring the concentration of gluten antigens below a threshold of tolerance, and so help CD patients tolerate a gluten-low diet, if not replace a gluten-free diet. Clinical trials are needed to determine dose, safety and efficacy of a gluten-protease treatment based on AnP2 as a food additive. A clinical pilot study of AN-PEP was carried out with 16 CD patients, and it was concluded that AN-PEP treatment combined with gluten challenge was safe [36]. However, this study was inconclusive with regards to the efficacy of AN-PEP treatment, so other clinical trials are awaited [4].

Since AnP2 is proline-specific and as the TCC535.3.23 epitope QLQPFQPELPY contains four proline residues, it is a very efficient substrate for cleavage by AnP2. Pathogenic gluten epitopes cluster in regions with high content of

proline residues [2], and proline residues are abundant in gliadin-derived epitopes [24], so a proline-specific protease could conceivably be effective at preventing pathogenic gluten reactivity in CD patients. This was also indicated by previous studies using prolyl endo-peptidases (PEP) from bacterial sources [17,22]. Of further support is the finding that pre-treatment of food gluten with bacterial PEP showed beneficial effect in a gluten-challenge of CD patients [23]. In a rat model of gluten proteolysis, the barley-derived endoprotease EP-B2 was shown to accelerate gluten digestion *in vivo* [10]. A strategy of combining a PEP and EP-B2 in a drug compound called ALV003 has been investigated [9,26], and has been shown to reduce immune reactivity after gluten challenge in CD patients. Clinical symptoms were not significantly reduced after pre-treating gluten meals with ALV003 [37], but clinical phase 1 and 2 trials with ALV003 as a food additive have shown promising results [12,27]. Gluten-degrading enzymes from other sources such as germinating wheat [32] or oral bacteria [42] could also have therapeutic potential. Further studies are warranted to identify the best suited strategy for enzymatic detoxification of gluten. An advantage of AN-PEP is the low pH range of 2–8 with an optimum between pH 4 and 5, and resistance to low pH and pepsin degradation [34]. We found that AnP2 used in our study was stable at pH 2.5 to 7.5, and determined the pH range of AnP2 to be 3.5 to 5 with an optimum of 4 (Table 1).

Our study shows that detoxification of native or deamidated gluten peptides by treatment with an proline-specific endo-peptidase from *Aspergillus niger* (AnP2) prevents the reactivity of most gluten-reactive T cells derived from both children and adults with CD. There is likely a therapeutic benefit of AnP2 supplement to CD patients.

Conflict of interest

Erwin L. Roggen is employed by Novozymes, Bagsværd, Denmark. The remaining authors declare no conflicts of interest.

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