THE USE OF DIFFERENT PROTEASES TO HYDROLYZE GLIADINS

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ABSTRACT

Gliadins represent alcohol-soluble fraction of wheat storage proteins which is responsible for development of celiac disease. The only and effective treatment for celiac disease is strict adherence to a gluten-free diet excluding any food made with wheat, as well as rye, barley and possibly oat flour. Enzymatic modification of wheat gliadins seems to be an alternative method for decreasing of celiac activity. The aim of our study was a trial of enzymatic modification of wheat gliadins using fungal (Aspergillus spp., Aspergillus oryzae, Aspergillus niger) and bacterial (Bacillus licheniformis, Bacillus steaothermophilus, Bacillus thermoproteolyticus, Streptomyces griseus) proteases. The reaction was performed up to 60 min, stopped by addition of appropriate synthetic inhibitor and products of limited proteolysis were analyzed by SDS-PAGE method. From fungal proteases most effective proteolytic activity was observed using acid proteinase from A. niger since wheat gliadins and low molecular weight peptides were completely degraded. Bacterial proteases form B. licheniformis and B. thermoproteolyticus acted very effective and as the result of hydrolysis, the products of lower molecular weight (<15 kDa) occurred. Most of the wheat gliadins were susceptible to proteolysis by examined bacterial enzymes (exception were protease from B. steaothermophilus and S. griseus). Although wheat gliadins are susceptible to enzymatic degradation, further analysis (e.g. immunochemical or mass spectrometry) are desirable to confirm if the products of proteolysis have lost or at least partially decrease their celiac activity.

Keywords: Gliadins, proteases, celiac disease

INTRODUCTION

Wheat proteins play an important role in the determining both nutritional and functional properties of derived foods. Based on their different solubility, wheat proteins can be classified into water/salt-soluble albumins and globulins, alcohol-soluble gliadins, and the glutenins soluble in diluted acid/base solutions (Mammone et al., 2011). Wheat gliadins, traditionally subdivided into α-, γ- and ω-fractions according to their mobility in polyacrylamide gels, represent monomeric polypeptides with molecular weight approx. 28-50 kDa (Wieser, 2007; Vaccino et al., 2009). They are rich in glutamine (35%) and proline (15%) which makes them highly resistant to proteolytic degradation within the gastrointestinal tract causing the development of celiac disease (Gregorini et al., 2009). As a result, many long peptides (>20-mers) persisted during digestion in small intestinal lumen and are only partially hydrolyzed by brush-border membrane exopeptidases. These peptides (mostly 33-mer from α-gliadin) are deamidated by transglutaminase 2 (TG2) resulting in Th1 cell proliferation in the gut mucosa of celiac patients (Siegel et al., 2006). Thus eliminating the amount of Pro- and Gln-rich immunoexcitotoxic peptides in the small intestine can have a significant therapeutic potential.

Currently the only and effective treatment for celiac disease is a strict life-long gluten free diet forbids any foods containing wheat gliadins and corresponding proteins from rye, barley and possibly oat (Zingone et al., 2010). In recent years, several attractive therapeutic strategies have been arisen (see review Makkaria, 2014) from which enzyme therapy is proposed as one of the future options to reduce or even eliminate the celiac-active properties of gliadins. The enzyme therapy investigated two different approaches focused on microbial prolyl-endopeptidases (PEPs) (Martí et al., 2005; Stepniak et al., 2006; Ehren et al., 2009) and naturally-evolved glutenases (Stemman et al., 2009, 2010). The PEPs family has ability to cleave internal proline residues within a peptide sequence. Although, most of PEPs are irreversibly inactivated by gastric enzymes and acidic pH, it is unable to cleave some of Pro-rich peptides, such as the 33-mer from α-gliadin, it could simplify gluten structure and texture using encapsulation of PEPs in order to protect them (Solid, Khosla, 2005). On the other side, glutenases from germinating cereals evolutionarily selected for their cleavage of glutenine residues are also highly efficient in degradation wheat gliadins. In contrast to PEPs, naturally-evolved glutenases are resistant to digestion with pepsin and trypsin, and active over a large pH range. Further benefits are those they have a good safety profile, their isolation is very simple and they are widely used in malting industry for production of beer or baking ingredients (Stemman et al., 2009). Consequently, recent efforts focus on combination of appropriate PEPs with a naturally-evolved glutenases of complementary specificity.

In addition, other specific endopeptidases with cleavage activity for Pro-residues of different substrates have been reported, e.g. endopeptidase from Lactobacillus helveticus CNRZ32 (Chen et al., 2003), metalloendoprotease from Penicillium citrinum (Doi et al., 2004) and prolyl-endopeptidase from Aspergillus niger (Stepniak et al., 2006). The latter one is promising tool for celiac disease therapy.

In our study we try to find another appropriate microbial proteases of different origin (bacterial either fungal) suitable for wheat gliadin hydrolysis. The proteolytic conditions for each enzyme were determined according enzyme databases available for free via internet (BRENDA, MEROPS). However, large complexity and high proline abundance makes wheat gliadins quite resistant to proteolysis, therefore further analysis is required.

MATERIAL AND METHODS

Preparation of gliadins

Wheat gliadins were prepared by discontinuous fractionation of cereal protein complex in two-step extraction using 10 ml of solvent/g meal for 45 min under constant stirring at laboratory temperature. Extraction was carried out with 10% (v/v) NaCl to extract salt-soluble albumin/globulin fractions, and 70% (v/v) ethanol to extract gliadins. Each extraction step was repeated three-times following by centrifugation. Supernatant containing wheat gliadins was concentrated using vacuum distillation and lyophilized.

Enzymes used

Fungal proteases from Aspergillus sp., Aspergillus oryzae and Aspergillus niger were supplied by Fluka (Switzerland). Bacterial proteases isolated from Bacillus licheniformis (Sigma, USA), Bacillus steaothermophilus (Fluka, Switzerland), Bacillus thermoproteolyticus (Fluka, Switzerland) and Streptomyces griseus
Lyophilized wheat gliadins (1.5 mg) were dissolved in 150 μl of appropriate reaction buffer with optimal pH for each enzyme (see Tab 1). Enzymes were

<table>
<thead>
<tr>
<th>Protease</th>
<th>Reaction buffer</th>
<th>Reaction pH</th>
<th>Reaction temperature [°C]</th>
<th>Coenzyme</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC 3.4.21.63 (Aspergillus sp.)</td>
<td>0.1 M Tris-HCl</td>
<td>8.5</td>
<td>55</td>
<td>-</td>
<td>Pefablock&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>EC 3.4.24.39 (Aspergillus oryzae)</td>
<td>0.1 M acetate buffer</td>
<td>4.5</td>
<td>55</td>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>EDTA</td>
</tr>
<tr>
<td>EC 3.4.23.19 (Aspergillus niger)</td>
<td>0.1 M citrate buffer</td>
<td>3</td>
<td>55</td>
<td>-</td>
<td>Pepstatin&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>EC 3.4.21.62 (Bacillus licheniformis)</td>
<td>0.1 M Tris-HCl</td>
<td>9</td>
<td>70</td>
<td>-</td>
<td>Pefablock&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>EC 3.4.21.62 (Bacillus steaothermophilus)</td>
<td>0.1 M Tris-HCl</td>
<td>8</td>
<td>70</td>
<td>-</td>
<td>Pefablock&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>EC 3.4.24.27 (Bacillus thermoproteolyticus)</td>
<td>0.1 M Tris-HCl</td>
<td>8</td>
<td>70</td>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>EDTA</td>
</tr>
<tr>
<td>EC 3.4.24.31 (Streptomyces griseus)</td>
<td>0.1 M Tris-HCl</td>
<td>8</td>
<td>37</td>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>EDTA</td>
</tr>
</tbody>
</table>

<sup>1</sup> 4-(2-aminoethyl)-benzensulfonyl-fluoride, hydrochloride (AEBSF)
<sup>2</sup> (3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid

### RESULTS

Degree of hydrolysis and determination of molecular weights of peptides were done by SDS-PAGE under reducing conditions according to the Schägger-von Jagow method (Schägger and von Jagow, 1987).

**Proteolysis with fungal proteases**

Protease from *Aspergillus niger* (acid proteinase, aspergillopepsin II, EC 3.4.23.19) preferentially cleaved peptide bonds formed by amino acids Asn, Gln, Gly, Ala, Tyr and Thr. Serine endopeptidase isolated from *Aspergillus sp.* (generally called alkaline protease) belonging to the category EC 3.4.21.63 hydrolyze proteins with broad specificity but does not hydrolyze peptide amides. The last fungal protease used was metallopeptidase from *Aspergillus oryzae* (EC 3.4.24.39, also called deuterolysin) with capability to hydrolyze bonds with hydrophobic residues in P1’ (MEROPS, BRENDA).

Although all fungal proteases coming from the same genus: *Aspergillus*, they show different pattern of proteolytic activity on wheat gliadins as a substrate. Most effective proteolytic activity was observed using acid proteinase from *A. niger* since wheat gliadins and low molecular weight peptides were completely degraded (figure 1). In case of protease from *A. sp.* and *A. oryzae*, two products of <15 kDa were detected means that those examined endopeptidases are less efficient in cleavage of large peptides and intact proteins.

**Proteolysis with bacterial proteases**

Both serine proteases used are generally called subtilisins and has classification number EC 3.4.21.62, although coming from two different microorganisms: *Bacillus licheniformis* and *Bacillus steaothermophilus*. Subtilisins are non-specific peptidases with affinity for the hydrophobic amino acid residues in the peptide chain of substrate so they hydrolyze bonds formed by amino acids Ala, Leu, Ser, but also Val, Tyr, Phe, Gln, His (MEROPS, BRENDA). Another enzyme used in the study was metallopeptidase from *Bacillus thermoproteolyticus* (thermolysin, EC 3.4.24.27). Thermolysin has a high specificity for a hydrophobic amino acid residues, and hydrolyses mostly between residues P1-P1’, where P1 is the residue derived from any hydrophobic amino acid and P1’ is Leu, Phe, Ile or Val. Metallopeptidase from *Streptomyces griseus* (EC 3.4.24.31, commonly called mycolysin, pronase or actinase E), preferentially performs cleavage of peptide bonds with hydrophobic residues in P1’ (MEROPS, BRENDA).

The main subfraction of wheat gliadins is α-gliadin monomer with molecular weight of approx. 35 kDa and consisting from several celiac-active epitopes. During proteolysis with peptidase from *B. licheniformis* and *B. thermoproteolyticus* this fraction was easily hydrolyzed and almost completely degraded to low molecular weight peptides of <15 kDa. In case of serine

**Proteolysis**

Prepared in concentration 0.5 mg/ml in solution with the opposite pH level to reaction buffer (50 mM acetate buffer pH 5.5 or 0.1 M Tris-HCl pH 8.0). Proteolysis was performed in enzyme:gliadins ratio 1:100 for fungal proteases and 1:250 for bacterial proteases in temperature conditions appropriate for each enzyme. In case of metallopeptidases, coenzyme Zn<sup>2+</sup> in form of ZnCl<sub>2</sub> was added to the reaction mixture. Proteolysis was stopped after 15, 30 and 60 min with suitable inhibitor. The conditions for each protease are listed in Table 1.

![Figure 1](image1.png) Determination of molecular weights of wheat gliadins and degree of hydrolysis by SDS-PAGE after 60 min of proteolysis using specific fungal proteases isolated from *Aspergillus sp.*, *Aspergillus oryzae* and *Aspergillus niger*. Lane 1: wheat gliadins treated using fungal proteases; lane 2: un-treated wheat gliadins; M: molecular marker; vertical arrows indicate peptides after hydrolysis.

![Figure 2](image2.png) Determination of molecular weights of wheat gliadins and degree of hydrolysis by SDS-PAGE after 60 min of proteolysis using specific bacterial proteases isolated from *Bacillus licheniformis*, *Bacillus steaothermophilus*, *Bacillus thermoproteolyticus* and *Streptomyces griseus*. Lane 1: wheat gliadins treated using bacterial proteases; lane 2: un-treated wheat gliadins; M: molecular marker; vertical arrows indicate peptides after hydrolysis.
DISCUSSION

Nowadays celiac disease is one of the most prevalent food intolerance worldwide causing by ingestion of wheat gliadins and corresponding proteins of rye, barley and spelt. The disease is strict for celiac disease and results in lifelong gluten-free diet. Alternative strategies to completely destroy or at least partially decrease the celiac activity of wheat gliadins are the subject of extensive research. These include extraction of ancient wheat (van den Broeck et al., 2010), inhibition of trypsin/metalhioninase 2 (Maiuri et al., 2005), blocking of HLA-DQ2 to prevent binding of immunogenic peptides (Xia et al., 2007), prevention from immunogenic peptides through the tight junctions such as zonulin antagonists (Leffler et al., 2012), induction of tolerance using anti-cytokines such as anti-IFN-γ (Bethune et al., 2009) or anti-IL-15 (Yokoyama et al., 2009) and using gluten tolerant vaccines (Anderson, 2008). Finally, the most promising alternative therapeutic approach seems enzymatic cleavage of gliadins using specific prolyl-endopeptidases (PEPs) from different organisms, degradation of immunogenic peptides by germinating cereal enzymes and transamidation of gliadin peptides in celiac disease. The potential of protein hydrolysis and modification of prolamins by fungal endopeptidases (derived from Aspergillus and other fungal species) was demonstrated in our experiments. Fungal prolyl-endopeptidases due to their capability to explore proline-rich gluten peptides have been investigated by Shankar et al. (2004). Such enzymes, including those from Flavobacterium meningosepticum (FM), Sphinxomonas cupulata (SC) and Myxococcus sambus (MX), efficiently degrade an α2-gliadin-derived 33-mer peptide highly resistant to digestion and could serve as oral supplementation. FM enzyme showed a preference for Pro-Gln bonds, SC cleaved both Pro-Gln and Pro-Tyr bonds with comparable efficiency, and MX had a modest preference for Pro-(Tyr/Phenylalanine) sites over Pro-Gln sites (Shan et al., 2004). These prolyl-oligopeptidases are however not stable and functional under acidic conditions of the stomach and are unlikely to decay gluten epitopes before they reach the small intestine (Matsiysak-Budnik et al., 2005). In another study of Gass et al. (2007), a combination therapy of SC PEP and a gluten-specific cysteine endopeptidase (EP-B2) from barley was effective in degradation of clinically relevant quantities of gluten under gastric conditions. The intact gluten digested with the enzyme resulted in complete disappearance of 35 kDa wheat gliadins and peptides with low molecular weights products appeared. However further analysis of such products is desirable.

Alternative enzyme derived from fungus Aspergillus niger (AN-PEP) is active between pH 2-8, with an optimum activity at pH 4-5, and is therefore effective in the stomach conditions. The enzyme is also not degraded by pepsin and is stable functional under acidic conditions of the stomach and are unlikely to decay gluten epitopes before they reach the small intestine (Matsiysak-Budnik et al., 2005). In another study of Gass et al. (2007), a combination therapy of SC PEP and a gluten-specific cysteine endopeptidase (EP-B2) from barley was effective in degradation of clinically relevant quantities of gluten under gastric conditions. The intact gluten digested with the enzyme resulted in complete disappearance of 35 kDa wheat gliadins and peptides with low molecular weights products appeared. However further analysis of such products is desirable.

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MEROPS: The Peptidase Database, available at [http://merops.sanger.ac.uk](http://merops.sanger.ac.uk)