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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 sp.*, *Aspergillus oryzae*, *Aspergillus niger*) and bacterial (*Bacillus licheniformis*, *Bacillus stearothermophilus*, *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. stearothermophilus* 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 (**Mamone *et al.*, 2011**). Wheat gliadins, traditionally subdivided into α/β-, 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 transglutaminaze 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 immunotoxic 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 **Makharia, 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) (**Marti *et al.*, 2005; Stepniak *et al.*, 2006; Ehren *et al.*, 2009**) and naturally-evolved glutenases (**Stenman *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 PEP/s in order to protect them (**Solid, Khosla, 2005**). On the other side, glutenases from germinating cereals evolutionarily selected for their cleavage of glutamine residuses 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 (**Stenman *et al.*, 2009**). Consequently, recent efforts focus on combination of appropriate PEP/s 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**), metalloendoproteinase from *Penicillium citricum* (**Doi *et al.*, 2004**) and prolyl-endoprotease 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 stearothermophilus* (Fluka, Switzerland), *Bacillus thermoproteolyticus* (Fluka, Switzerland) and *Streptomyces griseus*

(Fluka, Switzerland) formed the second group of enzymes used in this study. All commercially available enzymes were selected for their proteolytic activity to cleavage peptide bonds in various substrates. Specific conditions for each enzyme were determined according on-line enzyme databases (BRENDA, MEROPS).

Proteolysis

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 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^{2+} in form of $ZnCl_2$ 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.

Table 1 Summary of proteolytic conditions

Protease	Reaction buffer	Reaction pH	Reaction temperature [°C]	Coenzyme	Inhibitor
EC 3.4.21.63 (*Aspergillus sp.*)	0.1 M Tris-HCl	8.5	55	-	Pefablock[1]
EC 3.4.24.39 (*Aspergillus oryzae*)	0.1 M acetate buffer	4.5	55	Zn^{2+}	EDTA
EC 3.4.23.19 (*Aspergillus niger*)	0.1 M citrate buffer	3	55	-	Pepstatin[2]
EC 3.4.21.62 (*Bacillus licheniformis*)	0.1 M Tris-HCl	9	70	-	Pefablock[1]
EC 3.4.21.62 (*Bacillus stearothermophilus*)	0.1 M Tris-HCl	8	70	-	Pefablock[1]
EC 3.4.24.27 (*Bacillus thermoproteolyticus*)	0.1 M Tris-HCl	8	70	Zn^{2+}	EDTA
EC 3.4.24.31 (*Streptomyces griseus*)	0.1 M Tris-HCl	8	37	Zn^{2+}	EDTA

[1] 4-(2-aminoethyl)-benzensulfonyl-fluoride, hydrochloride (AEBSF)
[2] (3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid

SDS-PAGE

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**).

RESULTS

Proteolysis with fungal proteases

Protease from *Aspergillus niger* (acid proteinase, aspergillopepsin II, EC 3.4.23.19) preferentially cleaved peptide bonds formed by amino acid 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.

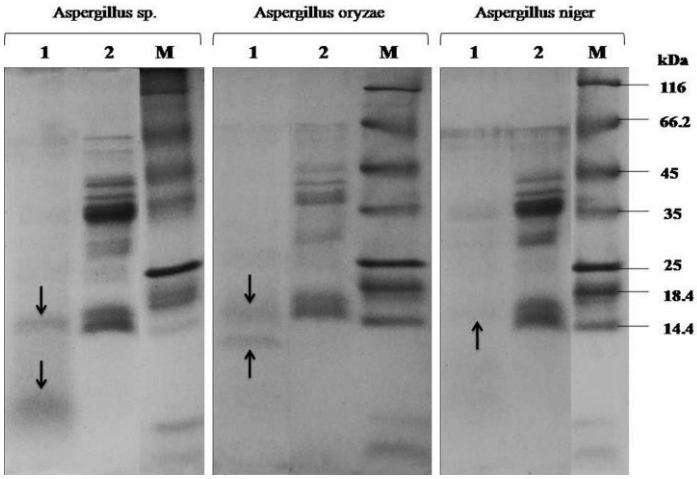

Figure 1 Determination of molecular weights of wheat gliadins and degree of hydrolysis by SDS-PAGE after 60 min of proteolysis using specific fungal proteases isolated from *Aspergillus sp.*, *Aspergillus oryzae* and *Aspergillus niger*. Lane 1: wheat gliadins treated using fungal proteases; lane 2: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

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 stearothermophilus*. 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

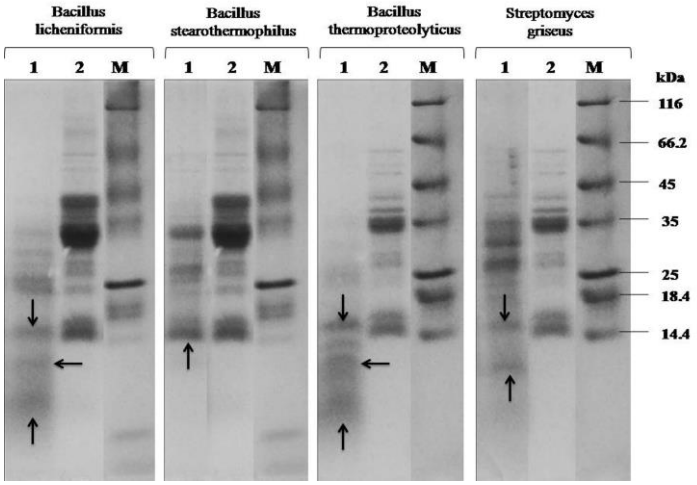

Figure 2 Determination of molecular weights of wheat gliadins and degree of hydrolysis by SDS-PAGE after 60 min of proteolysis using specific bacterial proteases isolated from *Bacillus licheniformis*, *Bacillus stearothermophilus*, *Bacillus thermoproteolyticus* and *Streptomyces griseus*. Lane 1: wheat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

peptidase from *B. stearothermophilus* and metallopeptidase from *S. griseus* the pattern of proteolysis was not obvious as compare to *B. licheniformis* and *B. thermoproteolyticus*. On the one hand fragment of approx. 15 kDa was occurred

(in case of *S. griseus* another fragment with <15 kDa was observed), on the other hand gliadins of 35 kDa were not completely hydrolyzed which means these two proteases act less effective. The degree of proteolytic degradation of wheat gliadins is shown in figure 2.

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 possibly oat. The only treatment for celiac disease is a strict 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 exploration of ancient wheat (**van den Broeck *et al.*, 2010**), inhibition of transglutaminase 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 tolerate vaccines (**Anderson, 2008**). Finally, the most promising alternative therapeutic approach seems enzymatic cleavage of gliadins using specific prolyl-endopeptidases (PEPs) of different origin, degradation of immunogenic peptides by germinating cereal enzymes and transamidation of cereal flour. In our work we have focused on cleavage capacity of novel specific endopeptidases of different origin (bacterial either fungal) which can serve as a potential tool to degrade wheat gliadins. Determination of protein hydrolysis and molecular weights of peptides was done by SDS-PAGE analysis.

Bacterial prolyl-endopeptidases due to their capability eliminate proline-reach gluten peptides have been investigated by **Shan et al. (2004)**.

Such enzymes, including those from *Flavobacterium meningosepticum* (FM), *Sphingomonas capsulate* (SC) and *Myxococcus xanthus* (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/Phe) 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 degrade gluten epitopes before they reach the small intestine (**Matysiak-budnik *et al.*, 2005**). In another study of **Gass *et al.* (2007)**, a combination therapy of SC PEP and a glutamine-specific cysteine endoprotease (EP-B2) from barley was effective in degradation of clinically relevant quantities of gluten under gastric conditions. Bacterial endopeptidases isolated from the genus *Bacillus* used in our study mostly hydrolyze bonds formed by amino acids Ala, Leu, Ser, but also Val, Tyr, Phe, Gln, His. Nevertheless, they are not classified into prolyl-endopeptidase enzyme family, 35 kDa wheat gliadins were susceptible to degradation, while low molecular weights products were 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 thus remains fully functional. Another advantage of this enzyme is capability to degrade both whole gluten and gluten peptides into non-immunogenic residues within minutes (**Stepniak *et al.*, 2006; Mitea *et al.*, 2008**). From our three examined fungal endopeptidases (derived from *Aspergillus sp.*, *Aspergillus oryzae* and *Aspergillus niger*), only the proteolysis using *A. niger* resulted in complete disappearing of 35 kDa wheat gliadins and peptides with low molecular weights means that those examined endopeptidase are very efficient in cleavage of large peptides and intact proteins in contrast to previous fungal enzymes. The same conclusion was achieved by **Stepniak *et al.* (2006)**. The authors measured efficiency of gluten degradation treated with AN-PEP and monitored the digestion process with mass spectrometry. Secondly, intact gluten digested with AN-PEP was separated by reverse-phase HPLC, than eluted fractions in specific time-points were separated onto SDS-PAGE gels followed by Western blotting with antibodies against gluten T cell epitopes. Proteolysis with AN-PEP resulted in complete degradation of both gliadin and glutenin molecules, and T cell epitopes in almost all cases. The data indicates that protease treatment, alone, should be considered as a potential tool for improving technological characteristics of flours.

CONCLUSION

The study was oriented toward proteolytic cleavage of wheat gliadins using microbial proteases. Gliadins were susceptible to proteolytic degradation of examined bacterial and fungal endopeptidases (exception were bacterial proteases from *B. stearothermophilus* and *S. griseus*), while products of low molecular weights (<15 kDa) were appeared. 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. All bacterial proteases of different origin showed slight differences in the degree of proteolysis. *B. licheniformis* and *B. thermoproteolyticus* acted very effective and as the result of hydrolysis, the products of lower molecular weight (<15 kDa) occurred. 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.

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MEROPS: The Peptidase Database, available at http://merops.sanger.ac.uk

BRENDA: The Comprehensive Enzyme Information System, available at http://www.brenda-enzymes.org/