

Review

Gluten proteolysis as alternative therapy for celiac patients: A mini-review

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Celiac disease (CD) results from damage to the small intestinal mucosa due to an inappropriate immune response to a cereal protein (wheat, rye, barley). The only treatment for CD is life-long avoidance of gluten proteins. Gluten-free products are not widely available and usually more expensive. That is why; there is an urgent need to develop an alternative therapy. Enzymatic degradation of gluten among other approaches, abolishing its immunogenic and toxigenic activities, is an attractive alternative strategy for oral therapy in CD. Several proteases following different approaches were studied. This review focuses on enzymes (microbial or vegetal) designed to digest gluten. Also, recent biotechnological procedures that use microorganisms (cell factories for enzymes) as starter culture to eliminate gluten are reviewed in this manuscript.

Key words: Celiac disease, gluten, proteolytic activity, lactic acid bacteria, therapy.

INTRODUCTION

Celiac disease (CD) is a chronic inflammatory disorder characterized by damage of the small intestinal mucosa caused by gluten proteins from wheat (the water-insoluble storage proteins) and similar proteins of barley and rye in genetically susceptible subjects (Mäki and Collin, 1997; Fasano and Catassi, 2001; Di Sabatino and Corazza, 2009). The disease is characterized by severe, immune-mediated damage to the jejuna mucosa (subtotal villous atrophy), typically involves chronic diarrhea, abdominal distension, weight loss and malnutrition (Mäki and Collin, 1997; Holmes and Catassi, 1999; Green and Cellier, 2007).

Gluten is a mixture of related proteins which are soluble in alcohol-water mixture (prolamins) and the glutenin which are insoluble polymers stabilized by interchain disulphide bonds (Wieser, 2007). During dough mixing, wheat flour is hydrated and as a result of the mechanical energy input discrete masses of the gluten proteins are

disrupted (Goesaert et al., 2005). The gluten proteins are transformed into a continuous cohesive visco-elastic gluten protein network. These proteins are unique and cannot even be found in cereals closely related to wheat such as barley and rye. During dough fermentation, the gluten network plays a major role in retaining the carbon dioxide. Gas retention properties in turn determine loaf volume and crumb structure of the resulting bread (Goesaert et al., 2005).

Currently, CD may affect approximately 1% of the population, according to serologic population based studies (Fasano et al., 2003; Green, 2007). Such a rate establishes CD as one of the most common food intolerances (Fasano and Catassi, 2001) and its prevalence is apparently increasing (Green and Cellier, 2007). In fact, CD has been recognized in populations with high wheat consumption: wheat is one of the most consumed cereals (Tatham and Shewry, 2008). CD has a worldwide distribution, detected not only in Europe and countries populated by Europeans, but also in North Africa (Rätsch and Catassi, 2001). A high prevalence occurs in North African and Middle Eastern populations. It is not reported in black African people (Woodward, 2007). In Western Sahara, CD is a common disorder, where the prevalence was reported as 5.6% in the Saharawi childrens (Rätsch and Catassi, 2001). In Europe, Australia and North

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Abbreviations: CD, Celiac disease; tTG, tissue transglutaminase; PEP, prolyl endopeptidases; GCP, germinating cereal proteases; F, flavobacterium; Lb, lactobacillus.

America, the prevalence was estimated between 0.5 and 1% (Cataldo and Montalto, 2007). In Asia, recent report from Hangzhou in China also suggested that the prevalence of adult CD may be more common in China (Freeman et al., 2010) than previously appreciated (Jiang et al., 2009). Of note, celiac disease has also been reported in immigrants to Canada from China, Japan and South Asia, particularly from the Punjab region of India (Freeman, 2010)

In Tunisia, CD is frequent. Probably, the Tunisian diet, which consists mainly of bread, couscous and pasta, contains 25 to 30 g gluten daily. Gluten is introduced early in Tunisian infant's diet, occasionally as early as the first month of life (Mankai et al., 2006). Moreover gluten intake has increased because of its use in processed foods, especially fast foods. In addition, cereal grains are the "staple foods" of Tunisian diet population. The prevalence of celiac disease in the general population (in Tunisia) has not been previously investigated. In 2006, the prevalence of CD was 1/700 in a population of apparently healthy blood donors (Bdioui et al., 2006; kallel et al., 2009). The prevalence of celiac disease in Tunisian schoolchildren was estimated to be about 1/157; close to the European prevalence (Ben Hariz et al., 2007).

The only treatment for celiac disease is a gluten-free diet. This involves elimination of the grains containing gluten, wheat, rye and barley, as well as food products and additives derived from them including bread, biscuits, cakes, pizzas, pasta, sauces and gravy (Green and Jabri, 2003). In fact, gluten is used on many foods to confer properties such as emulsification, cohesiveness, viscoelasticity and foaming (Esteller et al., 2005; Dayab et al., 2006). CD treatment also requires avoiding other glutenous products like soaps and cosmetics (which can be ingested while bathing or kissing) and preventing cross contamination of safe foods through processing and preparation (Thompson, 2008). So, total avoidance is extremely difficult. Thus, new strategies are being actively pursued to find new treatments or to eliminate noxious prolamins from cereal grains. During the last eight years, many approaches based on gluten hydrolysis in order to detoxify harmful gluten peptides were investigated.

Recently, potential therapeutic maneuvers were well reviewed (Tennyson et al., 2009; Lerner, 2010). In this review, we summarize the current enzymatic strategies (microbial or vegetal) used to hydrolyze gluten.

PATHOGENESIS OF CELIAC DISEASE

Gliadins and glutenins both contain disease-activating proteins (Dewar et al., 2006). After ingestion of gluten, it is degraded to multiple segments. Several gluten epitopes are immuno stimulatory; some are more active than others. An immuno dominant peptide of 33 amino acids (residues 57 to 89) identified from an α -gliadin fraction has functional properties attributable to many

proline and glutamine residues (Shan et al., 2002). Proline gives the peptide increased resistance to gastrointestinal proteolysis and causes a left-handed helical conformation, which strengthens binding with human leukocyte antigens HLA-DQ2 and HLA-DQ8 molecules on antigen-presenting cells (Woodward, 2007). Furthermore, researchers report that multiple non-HLA genes contribute to the genetic risk for CD (Zhernakova et al., 2011; Freeman et al., 2011). Additionally, glutamine residues are a preferred substrate for tissue transglutaminase-mediated deamidation, which confers an enhanced immunogenicity (Di Sabatino and Corazza, 2009). This leads to T-cell proliferation and production of cytokines, particularly γ -interferon that appears to perpetuate damage and uptake of antigenic gluten (Schumann et al., 2008; Bethune et al., 2009).

Interestingly, those immunogenic peptides are proline (15%) and glutamine (35%) rich polypeptides that are at the base of two major steps in the celiac inflammatory cascade: 1. they confer resistance to enzymatic breakdown, since the human intestine lack prolyl endopeptidase who can readily cleave proline-rich immune-stimulatory gluten peptides and 2) the glutamine rich gluten peptides are an ideal substrate for deamination by the tissue transglutaminase (tTG), an ubiquitous connective tissue enzyme (Dieterich et al., 1997). The deamination is crucial for the stability and avidity of the presented peptide in the HLA-DQ (human leukocyte antigen) groove and recognition of T-cell epitopes (Lerner, 2010). tTG is the auto antigen against which the abnormal immune response is directed to (Reif and Lerner, 2004) and two main auto antibodies: anti endomysium and anti tTG are the most useful serological markers to screen for the disease (Shamir et al., 2002).

DETOXIFICATION OF GLUTEN PEPTIDES BY PROTEOLYSIS

Generally, two alternative hydrolysis philosophies exist: to hydrolyse toxic gluten peptides after ingestion, in the gastrointestinal tract (the medical approach) or to hydrolyse them prior to the gluten ingestion, and during food processing (the food technological approach) (Loponen, 2006) (Figure1). In fact, the detoxification of gluten by proteolysis is not a novel idea and neither is the use of more than one protease in an effective detoxification procedure. For instance, Messer et al. (1964) showed that crude papain (which contains several diverse proteolytic activities) could detoxify gluten, whereas one purified papain proteinase failed to detoxify it.

MICROBIAL ENZYMATIC SOURCE

Gluten degradation can be performed by prolyl endopeptidases (PEPs). These are proteases, found primarily in plants and microorganisms. Prolyl

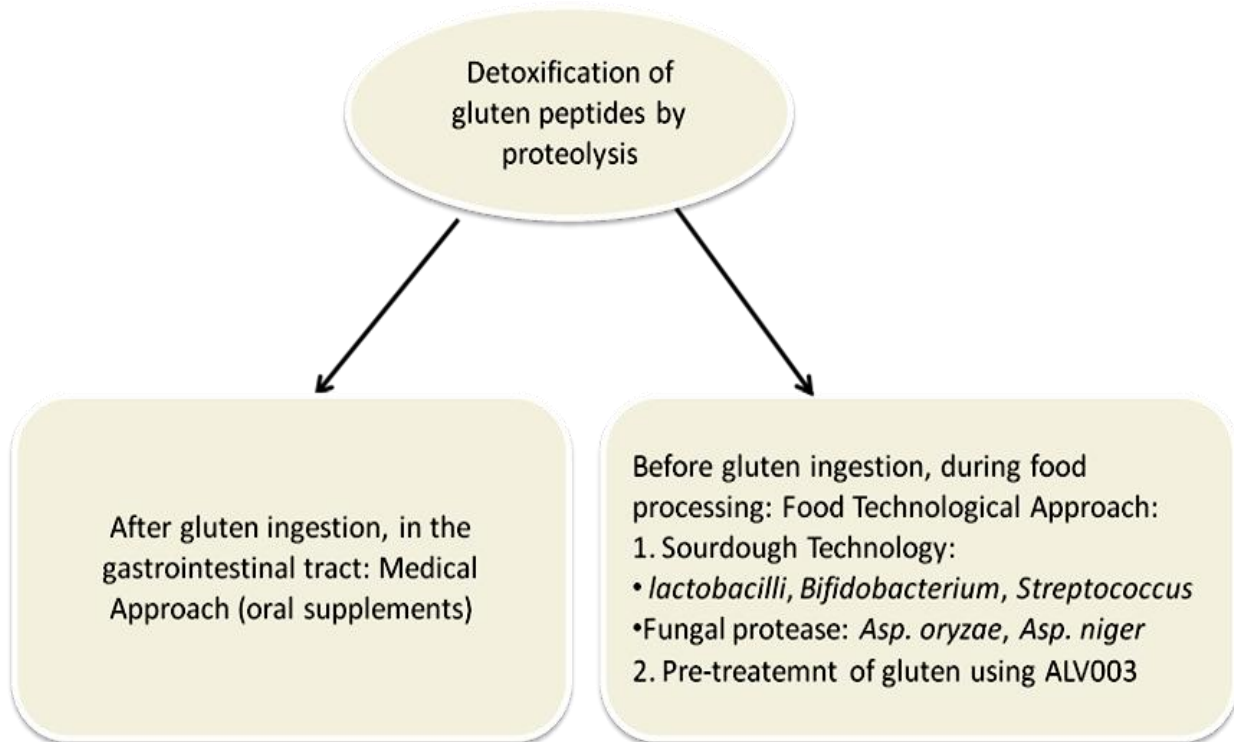


Figure 1. Two alternatives hydrolysis for gluten detoxification (ALV003 is a mixture of two glutenases; a cysteine endoprotease from germinating barley seeds (EP-B2) and a prolyl endopeptidase from *Sphingomonas capsulata* (SC-PEP); Asp: *Aspergillus*).

endopeptidases (PEPs) of microbial origin are endoproteolytic enzymes which, in contrast to human gastrointestinal protease, can readily cleave Pro-rich immunostimulatory gluten peptides (Hausch et al., 2002). This can be achieved by bacterial, or fungal enzymes that lend themselves to large-scale manufacturing (Piper et al., 2004; Stepniak et al., 2006).

A prolyl-endopeptidase produced by *Flavobacterium meningosepticum*, showed hydrolysing effect on a 33-mer peptide (the 33-mer was rich in proline: 13 residues and glutamine: 10), which is one of the most potent peptides involved in triggering the disease (Shan et al., 2002; Piper et al., 2004). The use of this endopeptidase has been proposed for an oral therapy for CD patients (Shan et al., 2002). *In vivo* studies with rats supported these findings, as the perfusion of PEP together with gluten peptides into the rat intestine accelerated the digestion of the gluten peptide *in vivo* by 50 to 100% (Piper et al., 2004). In a follow-up study, Pyle et al. (2005) showed that pre-treatment of gluten with PEP from *F. meningosepticum* avoided the development of fat or carbohydrate malabsorption in the majority of CD patients who ingested a low dose of a gluten supplement daily (5 g) during a challenge lasting 14 days. Similar properties (gluten detoxification) were obtained with PEP from *Myxococcus xanthus* and *Sphingomonas capsulata* (Shan et al., 2004; Gass et al., 2005) and *Lactobacillus*

helveticus (Chen et al., 2003).

Nevertheless, some contradictory results were noticed concerning PEP from *F. meningosepticum*. Matysiak-Budnik et al. (2004) showed that the hydrolysis of the 33-mer by PEP of *F. meningosepticum* in CD patients was not complete and led to the release of potentially immunogenic peptides. In addition, Shan et al. (2004) and Stepniak et al. (2006) reported that PEPs are inactivated by pepsin and acidic conditions in stomach. Therefore, Stepniak et al. (2006) introduced the use of a new enzyme; a prolyl endopeptidase from *Aspergillus niger* that was stable under gastric conditions (pH 2.0), optimally active at pH 4 to 5 and is completely resistant to digestion with pepsin, and efficiently degrades gluten proteins. Also, this PEP can be used as an oral supplement to reduce gluten intake in patients (Stepniak et al., 2006; Tennyson et al., 2009). This enzyme can be produced at low-cost at food-grade quality in an industrial setting (Edens et al., 2005).

CEREAL PROTEASE (PROTEASES FROM GERMINATING CEREALS)

The role of the proline and glutamine-rich storage proteins of cereals is to supply the embryo with nitrogen and amino acids during the first period of seedling

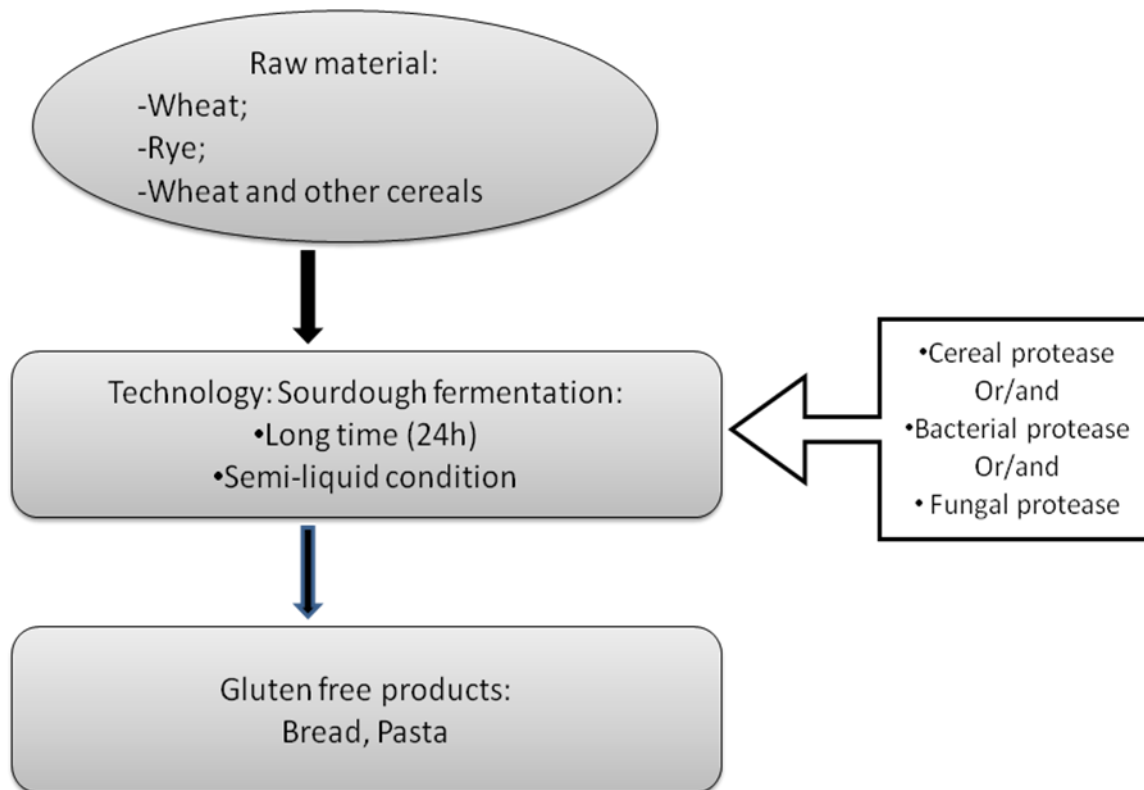


Figure 2. Proteolysis by sourdough fermentation: a tool for making gluten free products.

development. Therefore, it is likely that endogenous cereal proteases synthesized during germination (GCP: germinating cereal proteases) would be capable of extensively hydrolyzing these proteins. Many studies have checked this approach. Hartmann et al. (2006) showed the ability of proteases, isolated from wheat, rye and barley to degrade gliadin-peptides toxic for celiac patients. Results show that GCP were able to degrade intact gluten and celiac toxic peptides. These authors assumed that GCP are active in the stomach during digestion of food and also in the small intestine.

Bethume et al. (2006) showed gliadin hydrolysis (the 33-mer peptide) by EP-B2, a barley cystein proteinase responsible for hydrolyzing the bulk of the hordeins during barley germination. To facilitate gluten degradation, a two-enzyme cocktail, consisting of a glutamine-specific cysteine protease derived from barley B2) and a bacterially derived PEP (from *S. capsulata*), was developed (Siegel et al., 2006; Gass et al., 2007). The enzyme cocktail was called 'glutenase'. The efficacy of this two-enzyme glutenase was verified in a rat model of gastric gluten digestion. By combining two enzymes with gastric activity, it should be possible to increase the safe threshold of ingested gluten, thereby ameliorating the burden of a highly restricted diet for patients with celiac sprue. Recently, Tye-Din et al. (2010) reported that pre-treatment of gluten using glutenase (ALV003) can

abolish immune responses induced by gluten in patients (*in vivo*) with CD for three days.

PROTEOLYSIS BY LACTIC ACID BACTERIA AS STARTERS FOR SOURDOUGH FERMENTATION: CEREAL FOOD PROCESS

Proteolysis by lactic acid bacteria has been suggested as a new tool for food processing for celiac persons (Di Cagno et al., 2002, 2004, 2008; Rizzello et al., 2006; Gobbetti et al., 2007).

The potential of sourdough lactic acid bacteria as source of proteolytic enzymes was investigated during the last years. Sourdough is a mixture of flour and water that is fermented with indigenous lactic acid bacteria and yeasts (De Vuyst and Neysens, 2005). The use of sourdough as a natural leavening agent in the modern biotechnology of baked goods is increasing, largely because of the metabolic activities of lactic microflora. The use of sourdough fermentation for gluten degradation is shown in Figure 2.

Lactobacilli have been shown to possess an outstanding potential in decreasing the CD-inducing effects of gluten (Rollán et al., 2005; Gobbetti et al., 2007; Corsetti and Settani, 2007). Di Cagno et al. (2002) demonstrated a considerable degradation of various Pro-

rich peptides, including the 33-mer peptide during sourdough fermentation by some lactobacilli species. This finding has been exploited to produce sourdoughs containing 30% of wheat flour and 70% of other (non-CD-inducing) flours such as oat, buckwheat and millet, started with selected lactobacilli and fermented for 24 h (under specific processing conditions: long-time and semi-liquid fermentation). Following this, the mixed starter composed of *Lb. alimentarius*, *Lb. brevis*, *Lb. sanfranciscensis* and *Lactobacillus hilgardii* was shown to almost completely hydrolyze gliadin fractions and consequently the resulting bread was tolerated by CD patients as shown by intestinal permeability challenge (Di Cagno et al., 2004). The type of bread was technologically suitable. The same approach as those described for sourdough wheat bread (Di Cagno et al., 2004) was adapted for pasta making. The same pool of selected sourdough lactobacilli (*L. alimentarius* 15M, *L. brevis* 14G, *L. sanfranciscensis* 7A and *L. hilgardii* 51B) was used to preferment durum wheat semolina under semi-liquid conditions (Di Cagno et al., 2005). After fermentation, the dough was freeze-dried, mixed with buckwheat flour at a ratio of 3:7, and used to produce the "fusilli" type Italian pasta at an industrial level. As shown by immunological analysis, the concentration of gluten decreased from 6280 to 1045 ppm (destructive efficacy 83%) in the pasta fermented with lactic acid bacteria. This value was higher than those recommended by the Codex. Two levels are distinguished by the Codex Alimentarius Commissions of the World Health Organization and the Food and Agriculture Organization of the United Nations; < 20 ppm for foods that are naturally free of gluten or < 200 ppm for foods that have been rendered gluten free (Gallagher et al., 2004).

Recently, the same research team listed in the foregoing (Rizzello et al., 2007) showed that selected sourdough lactobacilli (for high and complementary proteolytic activities), in combination with fungal proteases, decreased the residual concentration of gluten (*Triticum aestivum* flour) below 10 ppm during food fermentation. The gluten concentration was lower than the threshold level indicated by the Codex Alimentarius Commissions of WHO and FAO for the gluten-free foods. This sourdough was fermented for 48 h at 37°C with ten lactobacilli (*Lb. alimentarius* 15M, *Lb. brevis* 14G, *Lb. sanfranciscensis* 7A, *Lb. hilgardii* 51B and *Lb. sanfranciscensis* LS3, LS10, LS19, LS23, LS38, LS47) (each strain at 10⁹ CFU/ml of dough) and two proteases of *A. niger* and *A. oryzae*, that were routinely used for bakery applications. Proteins fractions (gliadin and glutenin) extracted from this sourdough were freeze dried and incubated with small intestine mucosa (*in vitro* organ culture) from six patients. None of the intestinal T-cell lines demonstrated immunoreactivity (no interferon production) on the contrary to the negative control (dough without a bacterial and enzyme inoculum).

The same approach was investigated by M'hir et al.

(2009) where a pool of selected three enterococci (each strain at 10⁹ CFU/ml of dough) (M'hir et al., 2008) and fungal proteases (*R. oryzae*) was used to hydrolyse wheat gluten during long-time fermentation (doughs were incubated for 48 h at 37°C). The residual gluten on sourdough started with enterococci was 1648 from 75 621 ppm (98% of the gluten was hydrolysed) as shown by R5 antibody-based sandwich and competitive enzyme-linked immunosorbent assay (ELISA). By adding fungal proteases, the residual gluten decreased to a concentration of 1106 ppm, higher than those requested by the Codex Alimentarius Commission.

Fungal proteases, routinely used as bakery improvers, are indispensable to start the primary proteolysis of gluten. Polypeptides of intermediate dimensions (4 to 40 amino acids), generated from the native proteins, are the substrates for secondary proteolysis by complementary peptidases of sourdough lactobacilli (De Angelis et al., 2010; Gänzle et al., 2008; Rizzello et al., 2007). A combination of sourdough lactic acid bacteria selected for high and complementary proteolytic activities and an external addition of two fungal protease preparations were shown to hydrolyse gluten (72 h at 37°C) of durum wheat to less than 20 ppm (De Angelis et al., 2010). Durum wheat is an important food crop of the Mediterranean area, not only because of the large acreage but also for its importance in the human diet (Flagella, 2006). Durum wheat is largely used for making pasta, especially in the European and North Africa countries. Bread, burghul and couscous are also manufactured with durum wheat in several countries.

Longtime fermentation of dough by selected lactic acid bacteria was also shown to be a potential tool to decrease the risk of rye contamination of gluten free products for celiac patients (De Angelis et al., 2006a; Rizzello et al., 2006).

Alternatively, probiotics have been demonstrated to degrade gluten during sourdough fermentation (De Angelis et al., 2006b, 2007). In fact, as reported by Gobbetti et al. (2010) probiotics are functional microorganisms that contribute to food tolerance through their enzyme portfolio. Functional microorganisms are used in novel strategies for decreasing phenomenon of food intolerance (gluten intolerance) and allergy. The probiotic VSL#3 preparation (VSL Pharmaceuticals, Gaithersburg, MD) (ca. 450 billion cells/sachet) used containing *Streptococcus thermophilus*, *Lb. plantarum*, *Lb. acidophilus*, *Lb. casei*, *Lb. delbrueckii* spp. *bulgaricus*, *Bifidobacterium breve*, *Bifidobacterium longum* and *Bifidobacterium infantis*. When VSL#3 was used as a starter for bread making, it caused a marked degradation of wheat proteins. Celiac jejunal biopsies exposed to the Peptic-Tryptic digest from the dough fermented by VSL#3 did not show an increase of the infiltration of CD3+ intraepithelial lymphocytes.

Loponen et al. (2007, 2009) used germinated grains (wheat or rye) as a raw material in sourdough

Table 1. Summary of studies using proteolysis to degrade celiac peptides.

Strategy used	Protease from microorganism and/ or cereal	Reference
Bacterial protease	<i>Flavobacterium meningosepticum</i>	Shan et al. (2002)
	<i>Myxococcus xanthus</i>	Piper et al. (2004)
	<i>Sphingomonas capsulata</i>	Gass et al. (2005)
	<i>Lactobacillus helveticus</i>	Chen et al.(2003)
Fungal protease	<i>Aspergillus niger</i>	Stepaniak et al. (2006)
Germinated cereal protease (GCP)	Wheat, rye and barley	Hartmann et al. (2006) and Loponen et al. (2007,2009)
Mixture: GCP and bacterial protease	GCP from barley (EP-B2) and PEP: <i>Sphingomonas capsulata</i> (SC-PEP)	Siegel et al. (2006), Gass et al. (2007) and Tye-Din et al. 2010
LAB used as « starter »	<i>Lactobacillus</i>	Di Cagno et al. (2002, 2004, 2008), Rollàn et al. (2005) and
	<i>Lactobacillus</i> + <i>Streptococcus</i> + <i>Bifidobacterium</i>	De Angelis et al. (2006,2007)
	<i>Enterococcus</i>	M'hir et al. (2008)
Mixture: fungal protease and bacteria used as « starter »	<i>Lactobacillus</i> + Po <i>Aspergillus oryzae</i> +Pn <i>Aspergillus niger</i> <i>Enterococcus</i> + S <i>Rhizopus oryzae</i>	Rizzello et al. (2007), Greco et al. (2011) and M'hir et al. (2009)

PEP, Prolyl endopeptidases; Po, purified protease from *Aspergillus oryzae*; Pn, purified protease from *Aspergillus niger*; S, supernatant containing protease of *Rhizopus oryza*

fermentation. The results show that prolamins, including gliadins, were extensively hydrolyzed. These examples of trends in food technology to use sourdough fermentation for hydrolysis of the cereal proteins were attractive alternatives strategies as reported by Cabrera-Chávez and Calderón de la Barca (2010). For the first time, wheat flour which was rendered gluten-free during sourdough fermentation and was shown to be not toxic after administration to CD patients. Patients showed normal values of hematology, serology and intestinal permeability (Di Cagno et al., 2010). Later, the safety of daily administration of sweet baked goods made of wheat flour extensively digested by lactobacilli and fungal proteases was evaluated within patients with CD (*in vivo*) for 60 days. These patients' did not show clinical

symptoms, neither an increase of anti-TG antibodies nor a modification of the architecture or the grade of inflammation of the intestinal mucosa (Greco et al., 2011).

CONCLUSION

CD involves a complex interplay between environmental, genetic and immunologic factors. Wheat gluten and related proteins lead to inflammation in the small intestine. Stress factors like gastrointestinal infections have been found to increase the risk of triggering CD. The only currently available treatment for CD is complete elimination of gluten from toxic cereals: wheat, rye and barley. They can be substituted by other grains

such as rice, corn, quinoa, amaranth, sorghum, oats without cross contamination (with toxic cereals) and buckwheat, which are found to be safe (Briani et al., 2008; Kemppainen et al., 2007; Saturni et al., 2010). Improvements of symptoms are generally seen within days to weeks after the initiation of gluten-free diet.

Alternative treatments, such as oral doses of microbial endopeptidases to degrade wheat peptides are under trials. Also, sourdough degradation of gluten proteins is an option for food processing that includes fermentation.

From the reported results, the gluten hydrolysis can be achieved by cereal or bacterial or fungal protease or the combination of them. Table 1 summarizes strategies that have been used for gluten hydrolysis. Fundamental studies

(sourdough fermentation during long-time, adding selected lactic acid bacteria, fungal proteases and germinated cereal protease) have revealed several attractive targets for gluten destruction and prevention of CD. This alternative food technology may provide the option to reduce or even eliminate the harmful prolamins from cereal grains. It will be interesting to see whether any of these will become reality in the coming years.

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