



TO WHOM IT MAY CONCERN

The effects of microbial transglutaminase on gluten and safety concern with regard to its use in food preparations for celiac disease patients.

Celiac disease (CD), the most common food-sensitive enteropathy in humans, is caused by the lack of oral tolerance to gliadins and glutenins, protein components of wheat gluten, as well as to related proteins of rye and barley. The general opinion about the gluten consumption for CD patients is that an amount between 10 and 50 mg/day (Catassi et al., 2007) should be acceptable for most adult patients. This might be different when "gluten-free" foods, that means a gluten content less than 20 ppm, have been prepared with the use of mTG, as this is believed to generate gluten molecules with enhanced immunogenicity (Dekking et al. 2008). Due to the enhanced immunogenicity, lower amounts of such food might be tolerated by CD patients.

CD is strongly associated with the presence of the human-leukocyte-antigen (HLA) class II genes encoding for DQ2 and DQ8 heterodimers. The involvement of the immune system is well documented in the pathogenesis of CD, as inappropriate activation of lymphocytes in the intestinal mucosa, triggered by gluten peptides bound to DQ2 and DQ8 heterodimers on the surface of antigen presenting cells, is commonly observed. T cells produce high levels of pro-inflammatory cytokines, dominated by interferon gamma, which are responsible for the mucosal damage. DQ2 and DQ8 molecules preferentially bind peptides containing negatively charged residues at specific 'anchor' positions. However, gluten proteins, characterized by a high content in glutamine and proline residues, do not have many acidic residues. This discrepancy was resolved by the finding that gluten becomes a better T-cell antigen following deamidation. To date most of T-cell gliadin epitopes have been identified following deamidation catalyzed by tissue transglutaminase (tTG), which converts specific glutamine residues into glutamic acid, thus increasing the affinity of peptides to both DQ2 and DQ8 molecules. Furthermore, proline residues protect against digestive proteolysis and direct tTGase-mediated deamidation of glutamines. Additionally, T-cells drive the activation and clonal expansion of B cells, which differentiate into plasma cells and produce anti-gliadin and anti-tTG antibodies. By interacting with the extracellular membrane-bound tTG, tTG-autoantibody deposits in the basement-membrane region might induce enterocyte cytoskeleton changes with actin redistribution and consequent epithelial damage. However, a role of antibodies in CD pathogenesis remains elusive, also considering that a certain number of CD patients are agammaglobulinemic. The mechanisms described above represent the adaptive arm of the immune response to gluten, whereas the activation of innate immune mechanisms is still under debate.

Accordingly, a lifelong adherence to a gluten-free diet is mandatory for CD patients and many gluten-free products have been developed so far. However, their manufacture often requires the application of technological strategies to improve rheological and functional properties of gluten-free bakery products. An alternative for improvement is the incorporation of microbial transglutaminase (mTG), which allows cross-linking of proteins that can substitute the gluten network for the bakery.

Recently, mTG was assumed to deamidate both synthetic and natural gluten peptides which were recognized by gluten-specific T cells (Dekking et al. 2008). Various questions arise from this work, essentially related to the reaction conditions: i; a very long incubation time (16 hrs) and the presence of acetic acid, as it has been reported that acetic acid causes chemical deamidation of gluten (Berti et al. 2007); ii; the use of gluten peptides, as chemical deamidation is favored when peptides but not the whole molecules of gluten are used as substrates; iii, the uncertain purity degree of the non-commercial enzyme preparation; iv, a different pattern of deamidated glutamines found in comparison to those resulting from tTG-mediated deamidation. On the basis of these

considerations, it can be argued that most of detected deamidation of gliadin peptides was chemically induced. When we performed a similar reaction with commercial mTG in water or in basic conditions, we did not observe any deamidating activity. Instead, in the very same conditions tTG caused deamidation (Gianfrani et al. 2007).

It has also been reported that mTG treatment can increase the cross-reactivity to gliadin of immunoglobulins A (IgA) from CD patients, whereas in the presence of cysteamine, an amine group donor, cross-reactivity is strongly decreased (Berti et al. 2007). These reactions were conducted at pH 8.0, a condition that we showed to favor transamidation but not deamidation, as instead suggested by the authors. Accordingly, the increased cross-reactivity can find an alternative explanation by assuming auto-crosslinking of mTG, as already reported for tTG. This is reasonable considering that more than 5% lysine residues are present in the aminoacid composition of mTG. Gliadins-mTG complexes may then be responsible of the increased recognition by sera of CD patients. On the other hand, the presence of an amine group donor decreased this reactivity, as a consequence of epitope masking of glutamine residues by cysteamine. Most important, IgA antibodies toward gliadins are useful for the diagnosis of CD, but they do not play any role in the pathogenesis of CD. From this point of view, mTG treatment cannot increase gluten toxicity.

It was also feared that mTG-treated proteins from non toxic cereals can be recognized by the immune system of CD patients. In particular, Cabrera-Chavez et al. (2008) found that mTG-treatment of dough made of wheat or maize flours produced breads with prolamins more immunoreactive toward IgA of CD patients than those of untreated dough. Again, the increased cross-reactivity of gliadin antibodies following mTG treatment may not be responsible of generating toxicity for CD patients. Moreover, it is widely accepted that toxic sequences are recognized by T cells and can be generated only from prolamins of wheat, barley and rye but not of rice and maize.

Finally, we demonstrated the cross-linking activity of mTG in physiological conditions on two different known immunostimulatory gliadin peptides and the absence of any deamidating activity on the same peptides (Gianfrani et al, 2007). Most important, treatment of flour with mTG in the presence of lysine methyl ester preventively blocked gluten toxicity *in vitro*.

In conclusion, I can confirm that the possibility of using mTG to block, rather than increase, gluten toxicity has been widely acknowledged by the world community of gastroenterologists and immunologists (Schuppan and Junker, 2007; Celiac Disease Working Group, 2008).

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Faithfully



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