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Immunological evaluation of the alcohol-soluble protein fraction from gluten-free grains in relation to celiac disease

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Celiac disease (CD) is a gluten-sensitive enteropathy with an immune basis. We established the immune reactivity of the alcohol-soluble fraction from two minor cereals (tef and millet) and two pseudocereals (amaranth and quinoa) which are believed to be nontoxic based on taxonomy. Grains were examined in intestinal T-cell lines (iTCLs), cultures of duodenal explants from HLA-DQ2$^+$ CD patients and HLA-DQ8 transgenic mice for signs of activation. Our data indicated that tef, millet, amaranth, and quinoa did not show any immune cross-reactivity toward wheat gliadin, and therefore confirming their safety in the diet of CD patients.

Keywords:
Celiac disease / Organ culture / Pseudocereals / T cells / Transgenic mice

Celiac disease (CD) is the most common food-sensitive enteropathy in humans with an incidence as high as 1/100-1/300 among the European and North American population [1]. The environmental factors involved in the pathogenesis of CD are well characterized. CD results from the lack of oral tolerance to gliadins and glutenins, which are the two protein components of wheat gluten. The role of genetic factors is also fundamental to the development of CD. CD is strongly associated with HLA class II genes that map to the DQ locus. The HLA-DQA1*05 and DQB1*02 alleles that code for the α- and β-chains of DQ2 heterodimer, respectively, are present in about 90% of CD patients [2].The majority of the remaining patients express the DQ8 heterodimer encoded by the DQA1*03 and DQB1*0302 alleles. This genotypic feature may provide an explanation for the inappropriate activation of intestinal CD4$^+$ T cells triggered by gluten peptides bound to DQ2 and DQ8 heterodimers on the surface of antigen-presenting cells in CD patients [2]. The mucosal intestinal lesion is mainly induced by the production of IFN-γ from these gluten-specific T cells [3]. In addition, gluten becomes a stronger T-cell antigen following deamidation by intestinal tissue transglutaminase (tTG) [4]. Notably, the spacing between the glutamine and the proline residues was shown to play an essential role in the site specificity of the deamidation reaction and could be predictive for the identification of toxic peptides in gluten, hordeins, and secalins [5]. Wheat, rye, and barley belong to the grass family (Gramineae) of monocotyledons (Liliopsida class). This family also includes oat and other cereals such as rice, millet, and tef which are generally considered nontoxic [6, 7]. Recently, the use of pseudocereals, in particular amaranth (Amaranthaceae family) and quinoa (Chenopodiaceae family), that belong to dicotyledons (Magnoliopsida class) have been considered for the preparation of gluten-free food products. However, the believed lack of toxicity for most of these cereals and pseudocereals was based on their taxonomical classification rather than a direct evaluation of their immunostimulatory activity.

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Abbreviations: CD, celiac disease; **iTCL,** intestinal T-cell lines; **MLN,** mesenteric lymph node; **PT,** peptic-tryptic; **tTG,** tissue transglutaminase

In the present study, we analyzed the immune activity for CD of the alcohol-soluble protein fraction extracted from two cereals, tef and millet, and two pseudocereals, amaranth and quinoa, in comparison to wheat gliadin.

To perform a comparative analysis of gliadins with closely related proteins belonging to the examined species, we focused on the ethanol-soluble protein fraction extracted according to the Osborne protocol, by analyzing the relative protein content and electrophoretic mobilities (Supporting Information; Materials and methods). The amount of protein of the alcohol-soluble fraction in tested grains was minor compared with the total amount of protein, with the exception of millet (Supporting Information Table 1). As shown in Fig. 1A, upper panel, the pattern of the examined protein mixtures was found to be different in comparison with wheat gliadins. In particular, millet, quinoa, and tef produced poor discrete protein bands below 14 kD, whereas amaranth essentially separated in one 21 kD band and two bands with mw lower than 14 kD. Most importantly, Western blot analysis confirmed the absence of cross-reactivity with gliadin for any tested protein fraction (Fig. 1A, bottom panel).

Since immune reactivity of gliadin greatly increases following deamidation by tTG [4], and consensus sequences for tTG-mediated deamidation have been identified [5], we searched for possible deamidation sites in the tested proteins. A sequence alignment of wheat α-gliadin (UniProtKB/TrEMBL accession no. Q9ZP09), corresponding to the most conserved among α-gliadin amino acid sequences retrieved from Swiss-Prot [8], pennisetin, a 27 kD prolamin from millet (accession no. A6N4D4), and zein-α (accession no. P24450) was performed; no sequences for the alcohol-soluble proteins from tef, quinoa, and amaranth were found in the database. The data shown in Fig. 1B showed seven putative deamidation sites on both zein-α and pennisetin, but only two and one sites on zein-α and

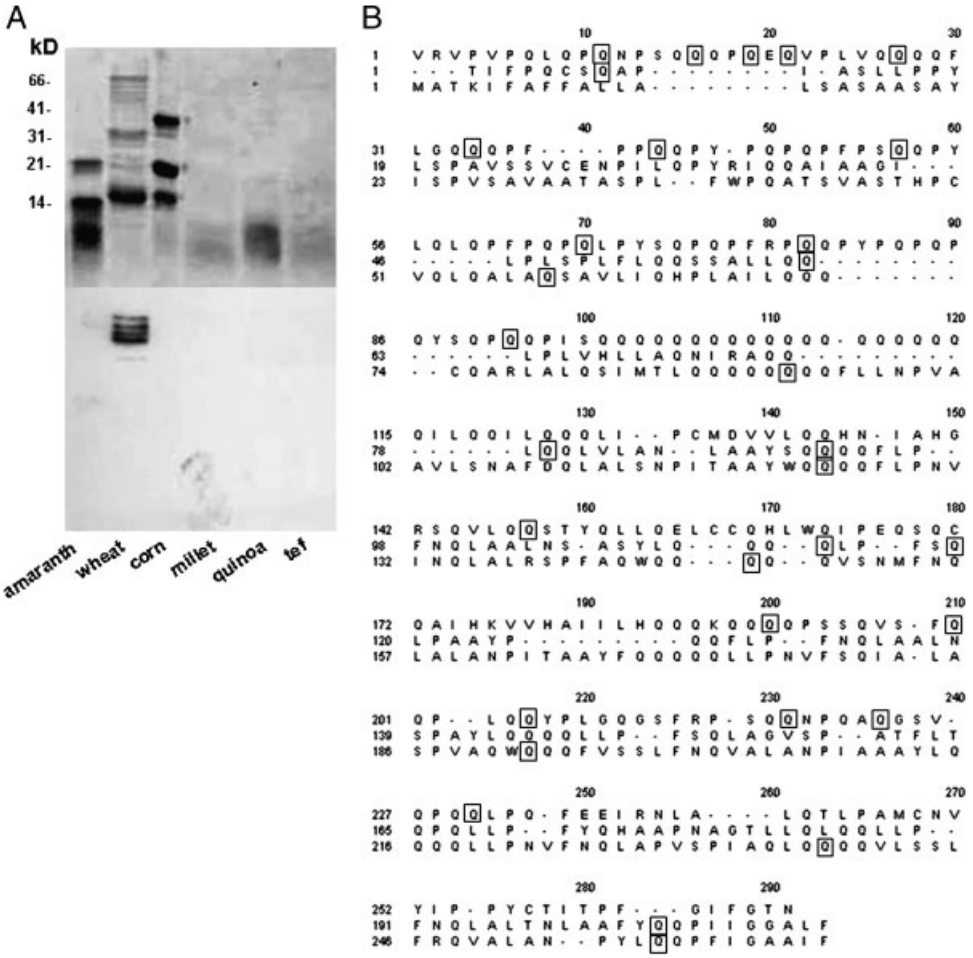

Figure 1. (A) Electrophoretic analysis of alcohol-soluble proteins. Coomassie blue staining of alcohol-soluble protein fractions isolated from the different flours (upper panel) and Western blot analysis of equivalent protein amounts with antigliadin antibodies (bottom panel). (B) Sequence alignment of alcohol-soluble proteins. Wheat α-gliadin (UniProtKB/TrEMBL accession no. Q9ZP09, upper), pennisetin (A6N4D4, middle), and zein-α (P24450, bottom) were aligned according to the Clustal method. The boxed Q's represent putative deamidation sites for tTG.

pennisetin, respectively, matching with the α-gliadin sequence. However, deamidation did not generate any putative epitope for DQ2 [9, 10] or DQ8 [11], with the exception of the sequence 111–123 (QQQQLPFSQLPA) of pennisetin that fulfilled the requirement for DQ8 binding [11]. As expected, no sequences of zein-α and pennisetin matched with known α-gliadin epitopes [12] and recently identified ω-gliadin, hordein, and secalin epitopes [13]. In addition, we compared the glutamine and proline content of amaranth (alcohol-soluble fraction) [14], quinoa (whole proteins) [15], and tef (whole proteins) [16] with gliadins [17] (Supporting Information Table 2). Pseudocereals and tef exhibited low levels of both amino acids, suggesting that tTG-mediated deamidation of glutamine residues in these proteins is unlikely.

A hallmark of CD pathogenesis is inappropriate $CD4^+$ T-cell activation in lamina propria (LP), triggered by deamidated gluten peptides bound to DQ2 and DQ8 heterodimers on the surface of antigen-presenting cells [2, 4]. Therefore, the use of intestinal T-cell lines (iTCLs) from CD patients has been shown to be instrumental in the analysis of the adaptive immune response in this pathology [18, 19]. In particular, the potential for the proteins characterized above to stimulate a gliadin-specific adaptive immune response was analyzed by testing iTCLs in the resting phase (Supporting Information; Materials and methods). A peptic-tryptic (PT)-digest of the alcohol-soluble proteins was treated with tTG under deamidating conditions before being incubated with iTCLs. IFN-γ production increased following incubation with a known stimulatory concentration of deamidated gliadin for each tested iTCL (Table 1); on the contrary, proteins from all other flours were ineffective in inducing IFN-γ production, both in their native form (data not shown) and after treatment with tTG. The statistical evaluation of the data confirmed no significant differences among the medians of examined grains with the negative control (Fig. 2A).

Next, the protein fractions were assayed in DQ8 tg mice, a well-established model of HLA-DQ8 restricted gliadin hypersensitivity, in which it is possible to reproduce some aspects of CD enteropathy [20]. Mice were mucosally immunized with PT-gliadin (Supporting Information; Materials and methods) that induced a significant response in cells that were stimulated in vitro with PT-gliadin from both the spleen and the mesenteric lymph nodes (MLNs) (Fig. 2B). Importantly, we did not detect any significant cell-mediated cross-reactivity with the tested proteins, as no sample was able to induce cell proliferation from splenic or MLN cells. Similar results were obtained at higher protein concentrations and when the corresponding deamidated proteins were analyzed (data not shown).

It is generally accepted that gluten contains not only peptides able to bind the pocket of HLA-DQ2/DQ8 molecules to elicit a specific T-cell response, but also peptides activating the innate immune system that co-operates to induce intestinal inflammation in genetically predisposed individuals [21]. In particular, these peptides induce a selective expansion of IELs and the rapid expression of CD25 by $CD3^-$ cells. Therefore, an ex vivo gliadin challenge of intestinal biopsies during a 24-h organ culture was used as valuable model to reproduce such early events occurring in CD (Supporting Information; Materials and methods). Consistent with the previous results [22], the number of cells/mm^2 expressing the CD25 activation marker was significantly higher in biopsy specimens cultured with PT-gliadin than in those cultured in medium alone (Fig. 2C). A trend of an increased density of $CD3^+$ IEL was also observed in these samples (Fig. 2C). On the contrary, compared with medium alone, no significant activation of lamina propria $CD25^+$ cells or increased intraepithelial $CD3^+$ cell number was noted in biopsy specimens cultured with a PT-digest from any of the ethanol-soluble proteins.

It is noteworthy that many grains besides wheat, rye, barley, and oats have not been subjected to immunological or feeding studies as in relation to their toxicity to CD patients. In particular, members of the grass family that are closely related to wheat, rye, and barley are considered toxic only on the basis of taxonomy. On the other hand, members belonging to other tribes like sorghum (Andropogonaceae), millet (Paniceae), Jobs tears (Maydeae), tef and ragi

Table 1. IFN-γ production in vitro of intestinal T-cell lines from CD patients following stimulation with the alcohol-soluble fraction from tested grains

	IFN-γ (pg/mL; mean ± SD)						
Patient no.	Medium	Wheat	Corn	Amaranth	Tef	Millet	Quinoa
1	45.0 ± 24.0	1586.4 ± 32.3	2.1 ± 0.1	39.3 ± 14.1	20.0 ± 5.1	5.0 ± 4.1	37.1 ± 25.2
2	10.9 ± 7.1	592.6 ± 10.4	145.6 ± 2.1	86.2 ± 14.5	154.4 ± 2.9	110.3 ± 37.8	110.0 ± 14.9
3	30.8 ± 18.5	1284.1 ± 26.7	0.2 ± 0.2	0.7 ± 0.7	0.6 ± 0.6	0.5 ± 0.5	105.2 ± 58.1
4	7.6 ± 4.5	1359.9 ± 201.8	23.1 ± 22.5	15.8 ± 0.6	28.5 ± 4.5	23.1 ± 3.2	9.4 ± 3.4
5	60.7 ± 1.3	139.8 ± 1.2	55.3 ± 6.4	49.4 ± 4.5	49.8 ± 8.9	66.2 ± 5.1	42.5 ± 6.4
6	0.2 ± 0.2	411.7 ± 123.7	0.5 ± 0.1	0.4 ± 0.4	0.2 ± 0.1	0.5 ± 0.1	0.2 ± 0.1
7	54.0 ± 0.2	6209.1 ± 784.9	54.1 ± 0.2	50.2 ± 1.0	74.3 ± 28.3	53.1 ± 2.0	50.2 ± 1.0

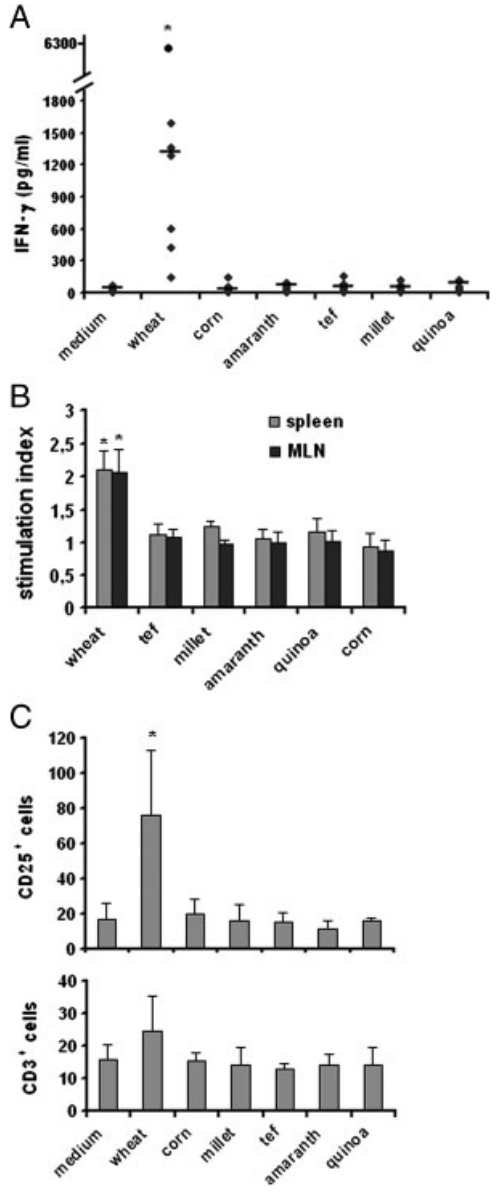

Figure 2. Immune effects of alcohol-soluble proteins isolated and digested from different grains on: (A) IFN-γ levels secreted by intestinal T-cell lines isolated from seven DQ2-positive celiac patients in vitro (Supporting Information Table 3). Bars represent median values; $^{*}p<0.05$, using the Kruskal–Wallis test. (B) Gliadin-specific response in DQ8 tg mice ($n=6$); following oral immunization with PT-gliadin along with CT, spleen and MLN cells were isolated and incubated in vitro with digested gliadin or alcohol-soluble proteins isolated from the different grains; the columns represent the proliferative response, expressed as a stimulation index from the mean ± SD of triplicate cultures, and are representative of three independent experiments. $^{*}p<0.05$ using the ANOVA test. (C) CD25 and CD3 marker expression following 24-h challenge of duodenal biopsies from seven treated CD patients with the alcohol-soluble protein fraction from tested grains; number of $CD25^{+}$ cells/mm^2 lamina propria and $CD3^{+}$/mm epithelium from the various experimental groups; columns represent mean values ± SD; $^{*}p<0.05$ using the ANOVA test.

(Eragrostideae), which appear to be related to corn, are considered safe. Analyses of amino acidic sequences were performed to support the toxicity conclusion of certain grains although even such studies are hampered by a lack of complete knowledge of all harmful sequences in wheat gluten proteins. Importantly, the parallel use of different CD models can provide crucial information before starting time-consuming and high-cost feeding studies for the assessment of gluten toxicity. Accordingly, we have analyzed the residual immunoactivity of the alcohol-soluble protein fraction of tef, millet, amaranth, and quinoa by using three different approaches: the challenge in vitro of deamidated gliadin-specific iTCLs; the ex vivo organ culture of duodenal biopsies from treated CD patients; and the activation in vitro of immune cells from gliadin-sensitized HLA-DQ8 tg mice.

In conclusion, our findings indicated that the presence of a substrate specificity for tTG was not a prerequisite for generating toxic peptides from grains. More importantly, tef, millet, amaranth, and quinoa did not show immune cross-reactivity with DQ2- and DQ8-restricted gliadin epitopes, or induction of early phases of immunotoxicity characteristic of CD. On the basis of our results, all tested grains may be considered suitable for use in the diet of patients with CD.

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