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Transglutaminase Treatment of Wheat and Maize Prolamins of Bread Increases the Serum IgA Reactivity of Celiac Disease Patients

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Celiac disease (CD) is mediated by IgA antibodies to wheat gliadins and tissue transglutaminase (tTG). As tTG is homologous to microbial transglutaminase (mTG) used to improve foodstuff quality, it could elicit the immune response of celiac patients. This study evaluated the reactivity of IgA of celiac patients to prolamins of wheat and gluten-free (maize and rice flours) breads mTG-treated or not. Prolamins extracted from wheat and gluten-free breads were analyzed by ELISA and immunodetected on membranes with individual or pooled sera from nine celiac patients recently diagnosed. Sera pool IgA titers were higher against prolamins of mTG-treated wheat or gluten-free breads than against mTG-untreated, mainly due to two individual patients' sera. The electrophoretic pattern of gluten-free bread prolamins was changed by the mTG treatment, and a new 31000 band originated in maize was recognized by three CD patients' IgA.

KEYWORDS: Transglutaminase treatment; immune response; Celiac disease; gluten free; prolamin

INTRODUCTION

Wheat gluten intolerance or celiac disease (CD) is an enteropathy triggered by ingestion of gliadins from wheat gluten and by prolamins from rye and barley (*1*). The pathogenesis of CD is mediated by IgA antibodies against native or deaminated peptides of gliadins and against the self-tissular transglutaminase (tTG), in an autoimmune response (*2, 3*). Until recently CD was considered to be a rare disease; however, nowadays it is recognized to be universally distributed, to include all races, with an estimated prevalence of 1–2% in the general population (*3, 4*).

CD in children under 2 years old is characterized by diarrhea, abdominal distension, and failure to thrive (*5*). However, in most cases, CD age-independent symptoms are less specific and include anemia, fatigue, weight loss, constipation, and even neurological anomalies (*6, 7*). When dietary gluten is removed, patients experience complete remission, and they relapse when gluten is reintroduced into the diet. Therefore, CD patients need a strict lifelong gluten-free diet, and a failure to comply with this requirement leads to a decrease in quality of life.

The food alternatives for CD patients include gluten-free products based mostly on maize, rice, and soy (*8, 9*). In wheat bread dough, gluten proteins naturally form a viscoelastic network required for the desired functional properties of bread products (*10*). Because these kinds of proteins are lacking (or in insufficient quantity) in maize and rice, the gluten-free breads lack good quality properties. Therefore, to improve the functional properties of gluten-free bread, the dough could be treated with microbial transglutaminase (mTG), which cross-links proteins and improves functionality (*11*).

Both tTG and mTG have the same type of activity and similar functional domains and secondary structures at their active sites (*12*). Thus, there is a latent risk in celiac patients to elicit or increase their immune response against the gluten-free bakery products made by mTG treatment. It could be due to several factors, including the mTG itself, the mTG−proteins complexes formed, or the peptides with modified glutamine residues (*13*).The aim of this study was to evaluate the immunoreactivity of IgA antibodies from celiac patients to prolamins from wheat and gluten-free breads (made with a mixture of rice and maize flours) produced before and after mTG treatment and to identify the responsible prolamins.

MATERIALS AND METHODS

Sample Preparation. Gluten-free (GF) bread was formulated according to a modification of the method of Moore et al. (*11*). GF dough was composed of 35 g of rice flour, 22.5 g of corn flour, 12.5 g of milk powder, 1.7 g of salt, 2 g of yeast, 1 g of sugar, 1 g of xanthan gum, 30 g of potato starch, and 105 mL of water. Wheat bread (WB)

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was made according to method 10-09 of the AACC (*14*), using 100 g of bread wheat flour, 2 g of salt, 2.1 g of yeast, 6 g of sugar, 3 g of vegetal butter, and 61 mL of water. Before the GF dough was mixed in a kneader (KitchenAid Classic) for 1 min, 300 ppm of mTG (Ajinomoto, activa TG) was added and mixed continuously for 2 min. WB dough fermentation was done according to the method of the AACC (*14*), and GF dough fermentation was carried out for 90 min at 30 °C and 85% relative humidity. After 20 min of baking at 200 °C, breads were ready and named GF+TG or WB+TG for gluten-free bread or wheat bread, respectively. Bread controls without TG were prepared similarly and called GF-TG and WB-TG. All of the products were frozen or freeze-dried until analyses.

Prolamin Extraction. Extraction of prolamins was carried out according to a modified method described by Gil et al. (*15*). Freeze-dried breads were ground, defatted with chloroform (5 g/25 mL) by stirring, and filtered on filter paper (Whatman no. 1) twice. Defatted samples were extracted twice for 1 h with 0.5 M NaCl (50 mL) and centrifuged at 2500*g* for 15 min. An additional water extraction was performed in the same manner. Precipitates were re-extracted using 70% ethanol, using the same 1:5 (w/v) ratio and a longer centrifugation (2500*g*, 30 min). Resultant prolamins were dialyzed against 1% acetic acid and freeze-dried.

Patients and Diagnosis Tests. Twenty-six patients with symptoms of probable CD or with a family history of CD were studied. Patients or their parents signed an agreement approved by the ethical committee of Sonora State Children′s Hospital (Hermosillo, Mexico). Intolerance symptoms, age of onset, and diet were registered by interview with patients or their parents.

A direct enzyme-linked immunosorbent assay (ELISA) was carried out to evaluate IgA antigliadins and antitransglutaminase, according to the method of Berti et al. (*16*). Briefly, microplates were coated overnight at 4 °C with 5 μg/mL gliadins or guinea pig liver transglutaminase (Sigma-Aldrich, St. Louis, MO) in coating buffer (0.1 M $NaHCO_3$, pH 9.6). After three washes with PBST (15 mM KH_2PO_4, 0.15 M NaCl, pH 7.4, containing 0.2% Tween 20), the plates were blocked for 1 h with 3% gelatin in PBS followed by three washes with PBST. The plates were incubated for 4 h at 25 °C with human serum samples (diluted from 1:100 to 1:12,800) in PBST containing 0.1% gelatin (PBSTG). Plates were washed in a similar way and incubated for 1 h with HRP conjugated antihuman IgA antibodies (DAKO, Carpinteria, CA) in PBSTG (1:2000 dilution), at 25 °C. After three washes with PBST, peroxidase activity was developed with 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma-Aldrich). The reaction was stopped after 5 min by the addition of 1 M H_2SO_4, and the absorbance at 450 nm was read (Microplate reader, Bio-Rad, Hercules, CA). Serum samples were individual serum from each celiac patient (CP), sera pool (all of the CP sera in equal proportion), and control was a sera pool from healthy people.

IgA Immune Response to Bread Prolamins. Prolamins extracted from GF or WB breads, mTG-treated or control, were dissolved in 70% ethanol. These solutions were used for serological tests by ELISA based on the Berti et al. (*16*) procedure, as described previously using bread prolamins instead of gliadins or transglutaminase, as antigens. Tested sera were the positive ones diagnosed as CD previously described. The same procedure was carried out using the sera pool. Percentage of immunoreactivity (% immunoreactivity) was calculated as

$$\% \text{ immunoreactivity} = (\text{AbsP}/\text{AbsW}) \times 100$$

where % immunoreactivity is the immunoreactivity expressed as percentage, AbsP is the absorbance at 450 nm of prolamins (measured in dilutions 1:100, 1:200, 1:400, and 1:800), and AbsW is the absorbance at 450 nm of gliadins from non-mTG-treated wheat bread (measured in dilutions 1:100, 1:200, 1:400, and 1:800).

IgA of sera pool to prolamins of wheat bread mTG-untreated was taken as 100% immunoreactivity as reference. Additionally, an internal control of the same sera against the same sample was assayed in each ELISA plate for percent immunoreactivity calculations.

Electrophoresis of Prolamins and Immunoblotting. SDS-PAGE was performed on 17% polyacrylamide gels according to the method of Laemmli (*17*) under denatured and reducing conditions. Gels were Coomassie blue stained or electrotransferred to nitrocellulose membranes by semidry blotting. After blotting, the membranes were blocked for 2 min with 0.05 M Tris, 0.15 M NaCl, 0.005 M NaN_3, pH 7.2, plus 2% Tween 20.

Detection of Prolamin Subunits Recognized by IgA from CD Patients. Detection of antigen on nitrocellulose membranes containing prolamins from WB-TG, WB+TG, GF-TG, and GF+TG was carried out according to the method of Calderón de la Barca et al. (*18*). Buffer for incubation and washing was TBST (0.05 M Tris, 0.15 M NaCl, 0.05% Tween 20, 0.005 M $NaNH_3$). The membranes were incubated overnight with human serum (1:50 v/v in TBST) at 4 °C. After three washes, 2 h of incubation was done with rabbit anti-human IgA antibodies (DAKO) diluted 1:1000 v/v in TBST. A third 1 h of incubation was done with AP-conjugated goat anti-rabbit antibodies (Bio-Rad) diluted 1:2000 in TBST. After the washings, AP activity was developed by incubating each strip in 1 mL of buffer A (0.1 M Tris, 0.5 mM $MgCl_2$, pH 9.5), 20 μL of NBT (0.3% w/v nitroblue-tetrazolium chloride in 70% v/v *N*,*N*-dimethylformamide), and 20 μL of BCIP (0.15% w/v 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt in dimethylformamide) according to Immuno-Blot Alkaline Phosphate Assay Kit instructions (Bio-Rad). After color development, the membranes were washed with distilled water and dried.

Statistical Analysis. Immunoreactivity was calculated from duplicates of four dilutions (100, 200, 400, and 800×) tested by ELISA. Mean values were compared by Tukey test using the statistical software NCSS, version 2001.

Table 1. Clinical Description of the Celiac Disease Patients (CP)

celiac patient	age (years)	antigliadin IgA titer[a]	antigliadin IgG titer[a]	anti-tTG IgA titer[a]	symptoms[b]
CP1	2.9	++++	++++	++++	D, AP, C, positive biopsy
CP2	16	+++	++++	+++	D, MA, UW, SH
CP3	8	++++	++++	++++	D, AP, F, SH, UW
CP4	3	++	++	++	F, UW, AP, A
CP5	1.1	++	+++	++	D, UW, F
CP6	1.7	++	+++	++	AP, Cn
CP7	2	++	+	++	AP, Cn
CP8	29	+++	+++	+++	MA, AP
CP9	13	++	++	++	D, F, AP, MA, SH

[a] The quantity of sings + mean the level of titers on IgA response. [b] D, diarrhea; AP, abdominal pain; C, cramp; MA, malabsorption; UW, underweight; SH, short height; F, flatulence; A, anemia; Cn, constipation.

RESULTS AND DISCUSSION

Effect of mTG on the Wheat and Gluten-free Breads. The gluten-free bread made from a mixture of rice and maize flours did not develop crumb, although Moore et al. (*9*) commented that it can develop a high volume. This can be due to insufficient hydrocolloids or additives on the dough formulation for gas retention during baking, related with crumb formation. Conversely, mTG addition to the gluten-free dough resulted in an acceptable crumb development of the bread, in the same conditions and composition as the former one except for mTG. In general, gluten-free breads treated with mTG tend to have a better overall quality due to the formation of a stable protein network (*11, 19*). In the case of wheat bread, addition of mTG caused uniformity on the bread crumb.

Diagnosis Test and IgA Immunoreactivity. Eleven of 26 patients' sera presented high positive titers of IgA against gliadins and tissue transglutaminase, and there were enough sera of 9 of them for the study. Their clinical description is summarized in **Table 1**. Intestinal biopsy to probe CD was taken in just one of the cases (CP1); however, patients with positive titers against gliadins and TG and whose symptoms were eliminated after the removal of gluten from the diets were also

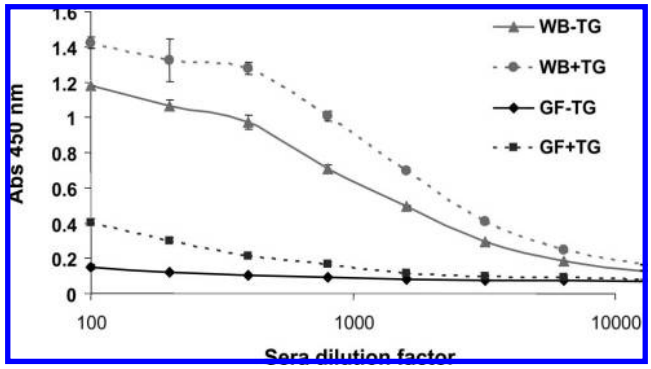

Figure 1. Immunological response of IgA from sera pool of celiac patients to prolamins from wheat and gluten-free breads, untreated (WB-TG and GF-TG) and mTG-treated (WB+TG and GF+TG).

Table 2. Immunoreactivity of Sera Pool and Two Celiac Disease Cases to Prolamins from Different Breads[a]

prolamin	% immunoreactivity of sera pool	% immunoreactivity of CP1 serum	% immunoreactivity of CP2 serum
WB-TG	100a	128.7 ± 9.6a	28.6 ± 7.3a
WB+TG	129.6 ± 9.8b	128.7 ± 10.0a	38.9 ± 12.8b
GF-TG	12.1 ± 1.2c	15.6 ± 3.4b	28.3 ± 5.7a
GF+TG	26.8 ± 5.6d	27.9 ± 5.1c	59.9 ± 10.0d

[a] Mean ± standard deviation. Different letters within the same column indicate significant differences at $p < 0.05$.

diagnosed as celiac disease patients (*20, 4*). All of the studied children filled these characteristics except for patient CP2, who presented strong responses to other prolamins that are different from these from wheat, rye, barley, or oat, as demonstrated in this study. All of the sera for experiments with prolamins were taken at the onset or diagnosis of the disease, before the change was made to gluten-free diets.

Figure 1 shows the IgA reactivity of the sera pool from nine patients to prolamins of the wheat and gluten-free breads, as Abs at 450 nm versus sera dilution factor. WB-TG prolamins produced a high IgA titer, whereas the reaction to GF-TG was negligible, as expected from a gluten-free product. However, the titers increased to the mTG treated of either WB or GF bread prolamins as shown in **Figure 1**.

In **Table 2** (column of sera pool), percent immunoreactivity against the prolamins of each bread type assayed is shown. Immunoreactivity was calculated from the ELISA data (as for **Figure 1**), taking as 100% the response of sera pool IgA against wheat bread prolamins as reference. Immunoreactivity of prolamins from WB increased approximately 30% after mTG treatment. This result is in agreement with the findings of Berti et al. (*21*) for isolated gliadins or digested gliadins treated with mTG in which the immunoreactivity of antigliadin IgA antibodies, with respect to non-mTG-treated, increased 85 and 30%, respectively. GF-TG prolamins induced a negligible reactivity (around 12%), whereas the IgA response to GF+TG was 15% higher. These results suggest that mTG treatment induces the formation of new epitopes on prolamins of wheat or gluten-free breads similar to the epitopes endogenously formed by tTG on peptides of prolamins inside the epithelial cells of celiac patients (*5*); otherwise, the IgA could not be able to recognize them.

Figure 2 presents the immunological reactivities to prolamins of two celiac patients' sera, which were extreme and quite different from each other, as examples of all nine tested sera samples. CP1 presented high titers but no differences between IgA reactivity to prolamins of untreated- and treated-mTG wheat

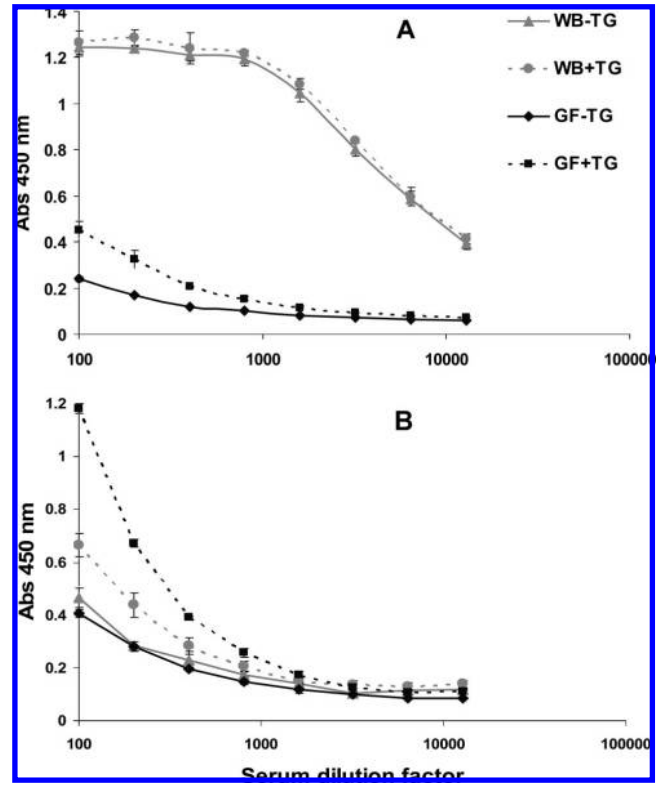

Figure 2. Immunological response of IgA from two celiac patients (**A**, CP1; **B**, CP2) to prolamins from wheat and gluten-free breads, untreated (WB-TG and GF-TG) and mTG treated (WB+TG and GF+TG).

bread (**Figure 2A**). With respect to the IgA reactivity of the same CP1 to gluten-free bread, there was a slight but significant ($p < 0.05$) increase from the mTG-treated bread (**Table 2**, column CP1). On the other hand, the reactivity of CP2 IgA to the prolamins (**Figure 2B**) was the same for mTG-untreated WB and GF prolamins. However, there was an increase in the reactivity of CP2 serum against mTG-treated GF prolamins (60% immunoreactivity), higher than that for the WB+TG (39%).

After each individual celiac disease serum had been analyzed for immunoreactivity, three of nine cases presented IgA against mTG-treated or untreated GF prolamins. Two of them presented higher titers against mTG-treated prolamins than these mTG-untreated. In the third case, immunoreactivity was the same (around 32%) for any of the analyzed prolamins of the four bread samples. Interestingly, patient CP3 with very high titers against gliadins and tTG (**Table 1**) did not present increased immunoreactivity either to prolamins from mTG-treated breads or to mTG-untreated GF prolamins.

Differences of the immunological responses of celiac disease patients to mTG-treated or untreated prolamins of breads can be due to an adaptive immunity, depending on environmental factors, which play a role in the pathogenesis of celiac disease (*22*). Thus, the presence of immunogenic sequences could have previously stimulated the adaptive immune system. CP1 was a small child at the onset of the disease with a more typical response, whereas CP2 was an adolescent who had suffered the illness as a child and his celiac disease diagnosis was recently made (**Table 1**). The third patient (CP8) with positive IgA reactivity to GF prolamins was an adult male. Probably CP2 and CP8 developed a new intolerance to other prolamins in addition to gliadins due to environmental factors that CP3 (an 8-year-old girl) has not been exposed to. According to Pastore et al. (*7*), if celiac patients do not follow a gluten-free diet, severe health complications can arise.

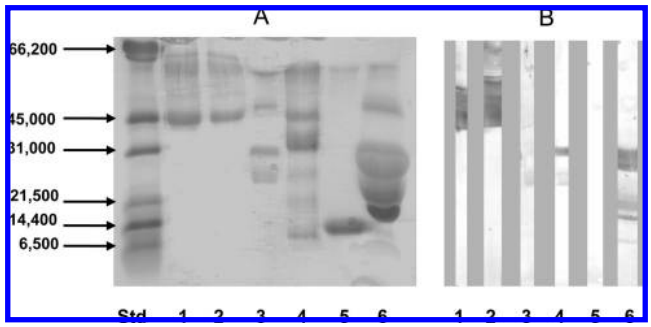

Figure 3. SDS-PAGE pattern of prolamins extracted from wheat and gluten-free breads and flours (**A**) and corresponding immunodetection on membranes (**B**) with sera pool from celiac disease patients. Samples: Std, molecular weight markers; 1, WB-TG; 2, WB+TG; 3, GF-TG; 4, GF+TG; 5, prolamins from rice flour; 6, prolamins from maize flour.

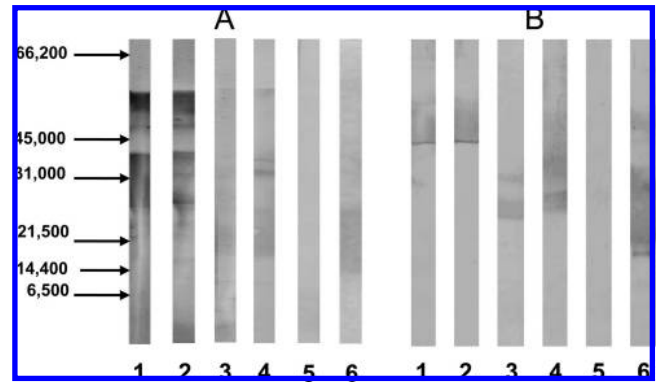

Figure 4. Immunodetection of prolamins on membrane with serum of two celiac patients (**A**, CP1; **B**, CP2). Prolamin samples: 1, WB-TG; 2, WB+TG; 3, GF-TG; 4, GF+TG; 5, rice flour; 6, maize flour.

Electrophoretic Pattern and Immunodetection of Prolamins. Electrophoretic patterns of prolamins from WB-TG, WB+TG, GF-TG, and GF+TG breads and the prolamins from raw rice and maize flours are shown in **Figure 3A**. Lanes 1 and 2 with WB-TG and WB+TG prolamins show typical patterns of gliadins with bands of molecular weights between 45000 and 66000 (*10*). The electrophoretic patterns shown are quite similar; apparently the wheat gliadins were not cross-linked by the mTG treatment, because glutenins, but not gliadins, are its preferred substrates (*23*).

The electrophoretic pattern of prolamins from GF-TG bread had two bands with molecular weights around 27500 and 34000 and two others of 46000 and 55000 (**Figure 3A**, lane 3), corresponding mainly to the molecular weight of total zeins from maize (*24*). The mTG treatment apparently cross-linked maize prolamins (zeins) because in lane 4 (**Figure 3A**) of GF+TG, there are several different bands from these of GF-TG, the strongest ones around 31000, 45000, and 55000. In addition to these described bands, maize contains zeins between 14400 and 31000 as shown in lane 6 for raw maize flour. Such zeins are very light in lanes 3 and 4 (from gluten-free breads), probably because these zeins are not easily extracted after heat treatment and aggregation induced by glucose (*25, 26*).

Immunodetection with sera pool of prolamins from different breads and flours is shown in **Figure 3B**. The bands on the blot appear to be more intense for WB+TG (strip 2) than for WB-TG (strip 1) in agreement with the results by ELISA (percent immunoreactivity, **Table 2**). This may be due to deaminated gliadins presenting new epitopes after the mTG treatment (*21*).

With respect to prolamins of gluten-free bread, GF-TG has a very weak band near 27300 (**Figure 3B**, strip 3). The most evident detection was for GF+TG with a band close to 31000 (**Figure 3B**, strip 4). Such a prolamin band of GF+TG may correspond to one of the several immunodetected on maize flour without mTG treatment (**Figure 3B**, strip 6). Previously, Kristjansson et al. (*27*), in agreement with the current results, found that some zeins induce an inflammatory reaction on the mucosa of some celiac patients. According to the overall results of reactivity the rice-containing products could be safer than the maize-containing products for some cases of celiac disease.

Immunodetection of prolamins extracted from wheat and gluten-free breads was also made using individual sera from each celiac patient; two cases are shown in **Figure 4**. The results show that differences between IgA reactivity of CP1 (**A**) and CP2 (**B**) to prolamins were not only in total percent immunoreactivity but also produced against prolamins of different electrophoretic patterns. IgA of CP1 recognized equally gliadins of untreated or treated with mTG wheat bread, showing reactivity against subunits from 28000 to 39000 and from 47000 to 56000 (**Figure 4A**, strips 1 and 2). On the other hand, IgA of CP2 mainly recognized gliadins (from WB-TG or WB+TG) of 44000−53000 (**Figure 4B**, strips 1 and 2, respectively).

Sera of CP1, who had the highest titer of IgA antigliadin and anti-TG (**Table 1**), did not clearly recognize GF-TG but slightly recognized GF+TG (**Figure 4A**, strips 3 and 4). Additionally, rice prolamins were not identified, but maize prolamins' frailty were recognized by IgA from CP1 (**Figure 4A**, strips 5 and 6). There was a strong response of IgA from CP2 against GF-TG, and it was even stronger against GF+TG prolamins (**Figure 4B**, strips 3 and 4). The more reactive was a 31000 band, which probably corresponds to an mTG-affected maize prolamin, because IgA from CP2 recognized several prolamins in raw maize flour (**Figure 4B**, strip 6).

It is known that several immunogenic peptides of wheat protein are rich in Pro and Gln residues (*28*), as the maize prolamins are. The amino acid sequence of maize prolamins contains Gln-Pro and Pro-Gln at the carboxy- and amino-terminal regions (*24*). Therefore, such ends could bind other proteins or free amino acids, as well as undergo Gln deamination, and the native sequence could be altered because of the mTG activity (*29, 30*), resulting in altered epitopes.

The mTG treatment of wheat and gluten-free breads increased the reactivity of IgA from some celiac patients' sera. The individual serum reactivity to prolamins of the tested breads was highly variable, although maize prolamins were clearly involved and those from rice were not. The results of this study constitute an indirect evidence of the risk of maize prolamins treated or untreated with mTG for some celiac disease patients, probably related to the use of maize in the Mexican diet.

In agreement with a previous study (*16*), it is suggested that IgA immunoreactivity can be used as a preliminary test of the safeness of a food product for celiac disease patients. Nevertheless, there is still a need to evaluate the risks associated with the consumption of the bakery products assayed in this study in in vivo or in-vivo-like models.

ABBREVIATIONS USED

tTG, tissue transglutaminase; mTG, microbial transglutaminase; CD, celiac disease; GF, gluten free; WB, wheat bread; GF+TG, mTG-treated gluten free bread; WB+TG, mTG-treated wheat bread; GF-TG, mTG-nontreated gluten free bread; WB-TG, mTG-nontreated wheat bread; CP, celiac patient.

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