Plant Foods Hum Nutr (2012) 67:24–30
DOI 10.1007/s11130-012-0274-4

ORIGINAL PAPER

Maize Prolamins Resistant to Peptic-tryptic Digestion Maintain Immune-recognition by IgA from Some Celiac Disease Patients

Francisco Cabrera-Chávez · Stefania Iametti · Matteo Miriani · Ana M. Calderón de la Barca · Gianfranco Mamone · Francesco Bonomi

Published online: 2 February 2012

Abstract Maize is used as an alternative to wheat to elaborate foodstuffs for celiac patients in a gluten-free diet. However, some maize prolamins (zeins) contain amino acid sequences that resemble the wheat gluten immunodominant peptides and their integrity after gastrointestinal proteolysis is unknown. In this study, the celiac IgA-immunoreactivity to zeins from raw or nixtamalized grains, before and after peptic/tryptic digestion was evaluated and their possible immunogenicity was investigated by *in silico* methods. IgA from some celiac patients with HLA-DQ2 or DQ8 haplotypes recognized two alpha-zeins even after peptic/tryptic proteolysis. However, digestion affected zeins after denaturation, reduction, and alkylation, used for identification of prolamins as alpha-zein A20 and A30 by MS/MS sequencing. An *in silico* analysis indicated that other zeins contain similar sequences, or sequences that may bind even better to the HLA-DQ2/DQ8 molecules compared to the already identified ones. Results concur to indicate that relative abundance of these zeins, along with factors affecting their resistance to proteolysis, may be of paramount clinical relevance, and the use of maize in the formulation and preparation of gluten-free foods must be reevaluated in some cases of celiac disease.

Keywords Celiac disease · IgA-reactivity · Zeins

Abbreviations

CD	Celiac disease
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase

Introduction

Celiac disease (CD) is an enteropathy that develops in genetically susceptible individuals by exposure to wheat gluten proteins, mainly the ethanol soluble fraction, called gliadins or collectively prolamins [1]. CD affects around 1% of the population worldwide, with classical symptoms as malabsorption, diarrhea, iron deficiency anemia, weight loss and other extra-intestinal symptoms [2].

CD goes into remission after dietary gluten withdrawal because it is primarily a T-cell mediated immune disease in which T cells recognize gluten peptides in the context of HLA-DQ2 or HLA-DQ8 molecules [1]. Such relatively large peptides are derived of prolamins from cereals as wheat, rye and barley, which have repetitive glutamine and proline-rich sequences. Therefore, these sequences are highly resistant to proteolysis by human gastrointestinal enzymes and gain access across the damaged intestinal epithelium in celiac patients [2]. In lamina propria, the prolamin peptides are deamidated

F. Cabrera-Chávez · S. Iametti · M. Miriani · F. Bonomi
Dipartimento di Scienze Molecolari Agroalimentari,
Università degli Studi di Milano,
Milano, Italy

A. M. C. de la Barca
Coordinación de Nutrición,
Centro de Investigación en Alimentación y Desarrollo,
Hermosillo, Sonora, México

G. Mamone
Istituto di Scienze dell'Alimentazione, CNR,
Avellino, Italy

A. M. C. de la Barca (✉)
Centro de Investigación en Alimentación y Desarrollo,
Carretera a la Victoria Km 0.6, P. O. Box 1735, Hermosillo 83000,
Sonora, México
e-mail: amc@ciad.mx

by tissue transglutaminase and activate the immune system leading to the development of CD [3].

After dietary gluten withdrawal, some CD patients still present symptoms; possibly, due to other proteins into the gluten-free diet that could trigger CD. For instance, there are some maize prolamins (zeins) which contain amino acids' sequences with high identity to immunodominant peptides from wheat prolamins [4]. Experimentally, maize zeins induced an inflammatory reaction in 50% of CD-patients by contact at the mucosa [5], and were recognized by IgA antibodies from other CD patients [6]. The results were obtained using undigested zeins obtained from raw flour but, the immunoreactivity could be highly reduced after processing and digestion [7].

Thus, the identification and characterization of immunoreactive zeins from processed maize after digestion are necessary to explore their actual participation in CD pathogenesis. In this study, the IgA immunoreactivity to zeins before and after processing and digestion using IgA antibodies from CD patients was evaluated and their possible immunogenicity was investigated by *in silico* methods.

Materials and Methods

Maize Prolamins Extraction and Digestion Commercial grains of raw and nixtamalized (lime-treated) white maize (*Zea mays*) were dried and ground before extraction [6]. The resulting maize flours were suspended in 70% ethanol (ratio 1:50, w/v) for 2 h at room temperature, and centrifuged at 2,500 × *g* for 1:50 min, the precipitate was freeze-dried. Prolamins digestion with pepsin from porcine gastric mucosa (Sigma P7012) with specific activity of ≥2,500 units/mg protein (1/100, w/w) was done in 0.1 N HCl, pH 1.8, incubating for 4 h at 37 °C [8]. After adjusting pH at 7.8 with 10 N NaOH, incubation with trypsin from bovine pancreas (Sigma T1426), with specific activity of ≥10,000 BAEE units/mg protein (1/100, w/w) was done for 4 h at 37 °C, followed by inactivation at 80 °C for 45 min. Enzyme activity of both pepsin and trypsin was evaluated by pH changes using a control of sodium caseinate. Digested prolamins were suspended in trichloroacetic acid (20%), stirred for 15 min, and centrifuged at 2,500 × *g* for 15 min, to precipitate peptides larger than 3 kDa which were dried. Additionally, commercial gliadins (Sigma Chemical Co., St. Luis, MO) were digested as described for maize prolamins.

SDS-PAGE of the native and digested zeins (from raw flour) was performed in a discontinuous gel system with 4% (upper) and 14% polyacrylamide [9] under reducing conditions. Gels of 10 wells each load 10 μg zeins were Coomassie Blue stained or electro-transferred onto nitrocellulose membranes.

Patients' Characterization Sera and blood samples were from 24 CD patients collected at the Sonora State Children Hospital and from private health services, after the protocol approval by the institutional ethics committee. The blood samples were typified for haplotypes after extraction of genomic DNA using the QIAamp DNA Blood Mini Kit (QIAGEN, USA) by conventional PCR (Bio-Rad MJ Mini Personal Thermal Cycler PTC 1148, USA) using specific primers [10].

The IgA anti-native and anti-digested zeins (from raw or nixtamalized grains) indexes were calculated for each patient [6] by a competitive enzyme-linked immunosorbent assay (ELISA). Briefly, microplates were coated with 100 μl of 5 μg/ml of each type of antigen (zeins) in 100 mM $NaHCO_3$, pH 9.6, overnight. After three washes with PBST (15 mM KH_2PO_4, 150 mM NaCl, pH 7.4, containing 0.2% Tween 20), the plates were blocked with 3% gelatin in PBST for 30 min at room temperature. Individual sera (1:100) were pre-incubated (4 h at room temperature) with digested gliadins (1 mg/ml in PBST + 0.1% gelatin). The previously coated microplates were incubated overnight with the pre-incubated human serum samples. The plates were washed three times with PBST and incubated with HRP-conjugated anti-human IgA antibodies (1:2000 dilution in PBSTG). After three washes, HRP activity was developed with 3,3′,5,5′-tetramethylbenzidine. The reaction was stopped with 1 M H_2SO_4, and the absorbance was read at 450 nm (Microplate Reader, Bio-Rad, Hercules, CA).

The serum reactivity of IgA anti-zeins was expressed as an index value, that is, the optical density of the test serum divided by the cutoff value. The cutoff value was the mean + 2 SD of the absorbance values of six negative sera of healthy donors. Index values of 1.0 and above were considered to be positive [11]. Sera from patients with positive IgA indexes of anti-native zeins were used to detect immunoreactive polypeptides that remained after the *in vitro* digestion of zeins. Additionally, an index value was calculated for IgA anti-digested gliadins in order to verify the effectiveness of the competitive step (pre-incubation of sera with digested gliadins).

Immunodetection Detection of zeins was done on nitrocellulose membranes [6]. Buffer for incubation and washing was TBST (0.05 M Tris, 0.15 M NaCl, 0.05% Tween 20, 0.005 M NaN_3). The membranes (two strips cut from each lane) were incubated overnight at 4 °C with human serum (1:50, v/v in TBST) from patients with positive anti-zein IgA indexes. Also, an immunodetection control was carried out using a sera pool from CD patients without IgA reactivity to zeins. After three washes, strips were incubated for 2 h with rabbit anti-human IgA antibodies (DAKO) diluted 1:1,000 (v/v) in TBST. A third incubation (1 h) was done with alkaline phosphatase-conjugated goat anti-rabbit antibodies (Bio-Rad) diluted 1:2,000 in TBST. After three washing steps, enzyme activity was developed by

incubating with 1 ml of 0.1 M Tris, 0.5 mM $MgCl_2$, pH 9.5, 20 μl of 0.3% w/v nitrobluetetrazolium chloride in 70% (v/v) aqueous N,N'-dimethylformamide, and 20 μl of 0.15% (w/v) 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt in N,N'-dimethylformamide, according to the kit instructions (Bio-Rad, Hercules, CA).

Mass Spectrometry Analysis Gels were distained with 50% acetonitrile (ACN) in 50 mM NH_4HCO_3, following reduction with 10 mM DTT (45 min at 56 °C) and thiol alkylation with 50 mM iodoacetamide for 45 min at room temperature. Each gel piece was further washed with 50 mM NH_4HCO_3, dried in ACN and vacuum-centrifuged prior to be taken up to 20 μl of 25 mM NH_4HCO_3 containing 12 ng/μl of trypsin from porcine pancreas (Sigma T6567). After ice incubation for 45 min, the excess of solution was removed, and another 20 μl of 25 mM NH_4HCO_3 were added. Digestion was left to proceed overnight at 37 °C. The resulting peptides were extracted with 50% ACN, 5% formic acid in three consecutive steps, with intermittent sonication. The peptide extracts were then combined and dried in a vacuum centrifuge. The resulting solids were re-suspended in 0.1% (v/v) aqueous trifluoroacetic acid and desalted on ZipTip C18 microcolumns (Millipore). MS/MS data were obtained on a Q-STAR spectrometer, equipped with a nanospray interface (Protana, Odense, Denmark). Proteins were sprayed from gold-coated medium length borosilicate capillaries (Protana) at a capillary voltage of 800 V. Multi-charged ion isotopic clusters were selected by the quadrupole mass filter and fragmented by collision (energy 20–40 eV, depending on the peptide size). Interpretation of the MS/MS spectra of the digests was done by using MS-Pattern (http://prospector.ucsf.edu.ucsfhtml4.0/mspatternhtm).

In Silico Analysis Zeins' sequences available in UniProtKB Data Bank were used to identify the possible peptides efficiently bound to HLA-DQ2/DQ8 molecules. The consensus approach was used to predict the best binders to class-II MHC (Alleles: HLA DQA1*0501-DQB1*0201; HLA DQA1*0301-DQB1*0302) on the basis of the implemented Consensus Percentile Rank [12]. The identity was estimated by the basic local alignment software BLASTP 2.2.25+ [13, 14].

Results and Discussion

Zeins' Digestion The electrophoretic patterns of native zeins and zeins after treatment with digestive-tract proteases (just from raw grains) are shown in Fig. 1. An important part of the prolamins fraction between 22 and 24 kDa resisted proteolysis and exhibited the typical alpha-zeins' pattern [15]. These alpha-zeins (~22 kDa) are more resistant to proteolysis than those under 18 kDa alpha-zeins [16]. According to Tschiersch et al. [17], resistance to proteolysis is not only due to the poor solubility of zeins but also by their relative low surface/weight ratio, in addition to their high content of glutamine and proline.

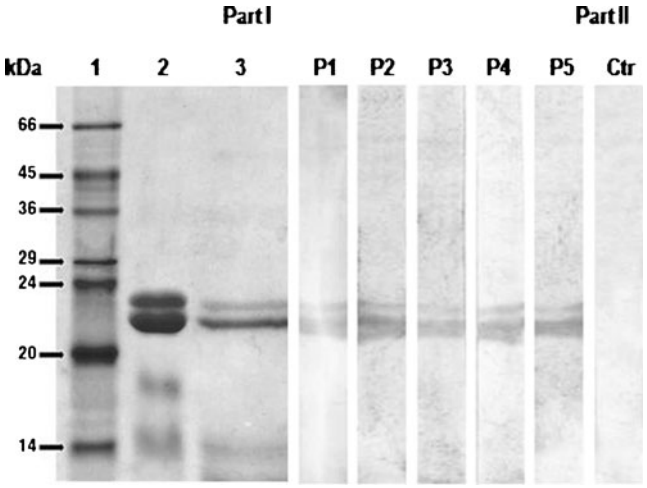

Fig. 1 SDS-Poliacrylamide gel electrophoresis (I) and immunodetection (II) of native and digested zeins. Part I, 1: molecular weight standards; 2: zeins; 3: zeins after digestion. Part II, membrane with zeins after digestion and incubation with IgA from CD patients. P1, P2, P3, P4, P5 are patients with positive IgA anti-zein indexes. Ctr: control of sera pool (IgA) from CD patients with negative titers to zeins

The first step in the zeins' digestion was pepsinolysis to simulate acid gastric digestion in mammals as in other studies [18]. Pepsin is an aspartic protease that cleaves proteins at leucine, phenylalanine and tyrosine residues and its hydrolysis is very fast unless hindered by the secondary or tertiary structure of the protein substrate [19], especially if there are beta-strands located along the perimeter of beta-sheets [20]. Although zeins contain the target amino acid residues for pepsin also present cysteine residues generating disulphide bonds that provide the resistance to its action [16]. Additionally, the content of beta-sheets and beta-turns in zeins decreases in acidic conditions (as during pepsinolysis) [21], impairing hydrolysis.

The low trypsinolysis during the second step of the simulated digestion could be due to the low content of cleavage sites on zeins in general. Trypsin cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline. Lysine and arginine content is very low in prolamins, in addition to their high content of proline, reducing susceptibility to hydrolysis by trypsin [22].

Although the simulated digestion assay does not exactly replicate the gastrointestinal processing, it represents a standardized proteolysis model. However, it seems to be more physiological than the exhaustive one by Berti et al. [7] where proteins' immunoractivity was highly reduced.

Patients' Characterization and Immunodetection From our group of 24 CD patients, five had positive and 19 had negative anti-zeins IgA indexes (IgA anti-native and anti-digested zeins, from raw and/or nixtamalized grains) (Table 1). Even more, the IgA indexes for each patient, measured at the time of active disease (before treatment), did not vary among the tested zeins. This result could indicate that most of the antigens remain after digestion and are not modified by nixtamalization, which is a strong processing. In addition, after a pre-incubation of the sera with gliadins the IgA indexes anti-zeins were still the same as before incubation, while IgA indexes anti-gliadins were negative. Therefore, the anti-zeins indexes are not due to cross-reactivity between them and gliadins which suggests the production of specific antibodies against zeins in CD patients.

The IgA reactivity against maize's zeins could be due to that some of their peptides are bound and presented by antigen presenting cells with HLA-DQ2/DQ8 molecules, which induces the immune response. As shown also in Table 1, three patients were HLA-DQ8 and two had HLA-DQ2. Although studied patients were from 1 to 65 years old, no young children presented IgA anti-zeins but an 8 year-old one, two adolescents and two adults CD patients. Possibly zeins are not involved in induction of inflammation in an early step to allow gliadins go across the epithelial mucosal barrier in predisposed individuals, but could induce immunogenicity in not-treated patients along the time. This idea agrees with results by Bergamo et al. [23], where only one of seven lines of intestinal T-cells from an Italian CD patient presented INF-γ production after stimulation with zeins.

IgA from individual sera clearly detected two bands in membranes with the prolamins transferred from electrophoresis gel. These bands correspond to zeins resistant to pepsin-trypsin digestion (Fig. 1, part II) and due to the electrophoresis profiles, these two bands are part of the alpha zein fraction [18].

Identification of Immunoreactive Proteins by MS The nature of the IgA-reactive bands in Fig. 1 (part I, lane 3) was investigated by MS/MS sequencing of tryptic-digests of the excised material. Although these zeins resisted proteolysis in their native state, after reduction and denaturation treatments before and after SDS-PAGE, were hydrolyzed by a longer trypsinolysis used to remove them from the electrophoretic gel.

Peptides were detected by MS, and fragmented by MS/MS analysis to obtain partial amino acid sequences. As shown in Table 2, sequences from the protease resistant bands in Fig. 1 (part I, lane 3), recognized by IgA of the patients' sera (Fig. 1, part II) were identified as alpha-zeins A20 and A30, respectively. However, A20 could be also alpha-zeins 19C1 or 19C2 because the detected sequence by MS corresponded to the same regions of the primary structure of these zeins. Vertical slab electrophoresis does not have enough resolution to separate this group of proteins [24]. Moreover, the identity estimated by basic local alignment indicates 98–99% of identity among alpha zeins A20, 19C1 and 19C2. Identified sequences corresponded to central regions of the primary structure of these zeins that have a theoretical mass consistent with their mobility in SDS-PAGE. It suggests that such zeins were not affected by the assayed proteolysis before electrophoresis or that proteolysis was restricted to the C- or N-termini.

In Silico Analysis Recognition of a peptide bound to the major histocompatibility complex or human leukocyte antigen (HLA) molecule is the critical step in activating T cells, and the binding is a necessary condition for the immune response activation [25]. Peptides to be bound to HLA-DQ2 or DQ8 molecules involved in CD can even be 9 amino acid residues long [26, 27].

Results of *in silico* analysis of zeins sequences that could be recognized by HLA-DQ2/DQ8 molecules are shown in Table 3. Both protease-resistant identified zeins had sequences that were included among good binders, although they were not the best sequences. The predicted output is given in units of IC_{50} nM. Therefore, a lower number indicates higher affinity. As a rough guideline, peptides with IC_{50} values <50 nM are considered as high affinity. Sequences

Table 1 Patients characterization: CD onset, haplotypes, anti-native and anti-digested zeins indexes for patients showing IgA reactivity to zeins

Patient	Age of onset (years)	Haplotype[a]	Symptoms[b]	Index of IgA anti-zeins (raw grains)		Index of IgA anti-zeins (nixtamalized grains)	
				Native	Digested	Native	Digested
P1	17	HLA-DQ8	D, MA, SH	4.50	4.84	4.43	4.53
P2	62	HLA-DQ2	D, MA, UW	3.73	3.90	3.85	3.67
P3	8	HLA-DQ2	D, SH, AP, UW	3.02	2.91	2.95	3.12
P4	16	HLA-DQ8	D, MA, SH, UW	4.10	4.07	4.23	4.02
P5	28	HLA-DQ8	D, MA, AP	3.39	3.77	3.50	3.54

[a] Allele tested by PCR were 0501 and 0301 in DQA1*, and 0201 and 0302 in DQB1*, corresponding to HLA DQ2 and DQ8, respectively. [b] *D* diarrhea; *MA* malabsorption; *SH* short height; *AP* abdominal pain; *UW* underweight

Table 2 Identification of immunoreactive zeins by MS/MS sequence analysis

Gel band[a]	Theoretical mass (kDa)	Identified sequence	Protein name	Accession number[b]
A	24.07690	^{58}LQQAIAASNIPLSPLLFQQSPALSLVQSLVQTIR ^{91}	Zeins alpha A20, 19 C1 or 19 C2	P04703, P06676 or P06677.
B	23.32505	^{87}VHLLAQNIR^{95} ^{22}TIFPQCSQAPIASLLPPYLSPAVSSVCENPILQPYR^{57} ^{58}IQQAIAAGILPLSPLFLQQSSALLQQLPLVHLLAQNIR^{95}	Zein alpha A30	P02859

[a] SDS-PAGE gel band (see Fig. 1)

[b] Swiss Prot accession number

identified in alpha-zein A20 were common to other zeins, as it was the case for the sequence found in alpha-zein A30 and haplotype HLA-DQ2. However, the sequence found in alpha-zein A30 was unique among those recognized by the haplotype HLA-DQ8. According to Skerritt et al. [28], the antibody reaction (in some celiac patients) against maize storage proteins did not simply result from cross-reaction of anti-gliadin antibodies as indicated by the competitive ELISA results. Thus, our data could indicate that zeins have a *bona fide* CD immunogenic capacity in some CD patients, probably derived from their sequences with high homology to some immunodominant peptides from gliadins [4]. Therefore, peptides' presentation by HLA-DQ2/DQ8 to the T cells could be similar to the gliadin's peptides. As a matter of fact, by basic local alignment we verify that the HLA-DQ2/DQ8 best binder peptides from alpha-zeins A20, 19C1, 19C2 and A30 had ~63% of identity to the 33-mer celiac immunodominant peptide reported by Shan et al. [29]. The exception was the best binder peptide to HLA-DQ8 from alpha-zein A30 that did not have identity to 33-mer, although it has 67% identity to a glutenin sequence identified as immunogenic [30].

In silico analysis essentially identified one region of alpha-zeins as a good binder of HLA-DQ2/DQ8 and two more sequences specific for HLA-DQ2 and -DQ8, respectively. According to experimental results by Tollefsen et al. [31], for alpha-gliadin, HLA-DQ2 and -DQ8-restricted T cells recognize sequences of different regions of the same protein (alpha-gliadin AJ133612). However, into a gamma-gliadin (M36999), the same peptide is recognized by both HLA-DQ2 and -DQ8 molecules.

Although the scores for maize prolamins in this study (Table 3) were similar among HLA-DQ2 and –DQ8 haplotypes, incidentally, the lowest scores (the best binders) for other alpha-zeins in our *in silico* analysis (data not shown) were found for the haplotype HLA-DQ2 (alleles DQA1*0501, DQB1*0201). Thus, a CD population with the haplotype HLA-DQ2 could be more sensible to peptides from zeins than those with HLA-DQ8. Kristjánsson et al. [5], found out a 6:10 ratio in an adult celiac population that was sensible to maize challenge in a contact probe test, whereas in our study the ratio was lower (5:24), although 19 of our cases were children under 7 years old. Different results could be also due to the genetics of population under the different studies, with Europeans mainly HLA-DQ2, while 12 of our 24 Mexican CD patients were HLA-DQ8.

As for the practical and clinical relevance of our findings, the *in silico* analysis identifies the theoretical best binder sequences for MHC (HLA-DQ2 and –DQ8) from different zeins. Therefore, an *in vitro* analysis is mandatory to verify the potential of the best binders to activate T cells in CD patients. If so, the presence of specific zeins in gluten-free foods could be a concern for both the food producer and the CD patients. It could be a screening method to look for

Table 3 *In silico* analysis. Best binders to the haplotypes HLA-DQ2 (Alleles HLA DQA1*0501-DQB1*0201 and HLA-DQ8 (Alleles HLA DQA1*0301-DQB1*0302)

Sequence	Position	Consensus percentile rank[a]	Zein name (Zein type)
Alleles HLA DQA1*0501-DQB1*0201			
TLLQLQQLLPFVQLA	1:208–222	10.12	Zein-alpha A20
TLLQLQQLLPFVQLA	1:208–222	10.12	Zein-alpha 19C1
TLLQLQQLLPFVQLA	1:208–222	10.12	Zein-alpha 19C2
FSQLPAAYPQQFLPF	1:138–152	10.17	Zein-alpha A30
Alleles HLA DQA1*0301-DQB1*0302			
LLQLQQLLPFVQLAL	1:209–223	10.06	Zein-alpha A20
LLQLQQLLPFVQLAL	1:209–223	10.06	Zein-alpha 19C1
LLQLQQLLPFVQLAL	1:209–223	10.06	Zein-alpha 19C2
VANAPTYLQQELLQQ	1:167–181	10.09	Zein-alpha A30

[a]Peptides with <50 in consensus percentile are considered high affinity

possible celiacogenic characteristics of proteins traditionally considered for gluten-free diets [32].

Aside from the occurrence of specific zeins in individual maize cultivars, resistance to proteolysis in the digestive tract appears to be of paramount relevance because preservation of the celiacogenic potential in some individuals. In this frame, the effects of food processing (different to nixtamalization) on the protease-resistance character may be worth investigating in further detail. The observation that these proteins resist tryptic proteolysis as extracted, but may be hydrolyzed once denatured and reduced—as indicated by our MS results—is indicative that process-related modification may have a role in determining their final properties.

From a clinical standpoint, several studies have shown that intestinal mucosa recovery after a gluten free diet may be incomplete and slow in some CD patients [33, 34]. According to Carroccio et al. [34], non-gluten peptides may be involved in the so called "refractory CD cases". At least two of the studied patients (P1 and P2) with positive anti-zeins IgA indexes remained positive (and clinical symptoms persisted) after placing them on a gluten-free diet including maize products.

Conclusions

Immunoreactivity of IgA from some celiac patients to prolamins from maize identified as alpha-zeins was the same before and after processing by nixtamalization either after *in vitro* digestion. IgA reactivity to maize prolamins could be due to a presentation of celiacogenic antigens in zeins. An *in silico* analysis confirmed that there are several peptides in maize prolamins sequences—including the A20 and A30 alpha zeins- that can be bound to the HLA molecules involved in CD pathogenesis.

Acknowledgement Authors are grateful to CONACyT (Mexican Council for Science and Technology) for providing a postdoctoral fellowship and for the partial financial support through the grant CB-2008/106227. The authors acknowledge the cordial support by N Sotelo-Cruz M.D. and M Ruiz-Dyck.

References

1. Abadie V, Sollid LM, Barreiro LV, Jabri B (2011) Integration of genetic and immunological insights into a model of celiac disease pathogenesis. Annu Rev Immunol 29:493–525
2. Caja S, Mäki M, Kaukinen K, Lindfors K (2011) Antibodies in celiac disease: Implications beyond diagnostics. Cell Mol Immunol 8:103–109
3. Briani C, Samaroo D, Alaedini A (2008) Celiac disease: From gluten to autoimmunity. Autoimmun Rev 7:644–650
4. Darewicz M, Dziuba J, Minkiewicz P (2007) Computational characterization and identification of peptides for *in silico* detection of potentially celiac-toxic protein. Food Sci Technol Int 13:125–133
5. Kristjánsson G, Högman M, Venge P, Hällgren R (2005) Gut mucosal granulocyte activation precedes nitric oxide production: Studies in coeliac patients challenged with gluten and corn. Gut 54:769–774
6. Cabrera-Chávez F, Rouzaud-Sández O, Sotelo-Cruz N, Calderón de la Barca AM (2009) Bovine milk caseins and transglutaminase-treated cereal prolamins are diferentially recognized by IgA of celiac disease patients according to their age. J Agric Food Chem 57:3754–3759
7. Berti C, Trovato C, Bardella MT, Forlani F (2003) IgA anti-gliadin antibody immunoreactivity to food proteins. Food Agric Immunol 15:217–223
8. Rakhimova M, Esslinger B, Schulze-Krebs A, Hahn EG, Schuppan D, Dieterich W (2009) *In vitro* differentiation of human monocytes into dendritic cells by peptic-tryptic digest of gliadin is independent of genetic predisposition and the presence of celiac disease. J Clin Immunol 29:29–37
9. Laemmli UK (1970) Cleavage of structural protein during assembly of the head of bacteriophage T4. Nature 227:680–685
10. Olerup O, Aldener A, Fogdell A (1993) HLA DQB1 and –DQA1 typing by PCR amplificaction with sequence-specific primers (PCR-SSP) in 2 hours. Tissue Antigens 41:119–134
11. Lagerqvist C, Ivarsson A, Juto P, Persson LA, Hernell O (2001) Screening for adult coeliac disease—which serological marker(s) to use? J Intern Med 250:241–248
12. Wang P, Sidney J, Dow C, Mothe B, Sette A, Peter B (2008) A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. PLOS Comput Biol 4: e1000048
13. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
14. Altschul SF, Wootton JC, Gertz EM et al (2005) Protein database searches using compositionally adjusted substitution matrices. FEBS J 272:5101–5109
15. Krishnan H, Kerley MS, Allee GL, Won-Seok K, Chunjiang JF (2010) Maize 27 kDa γ-zein is a potential allergen for early weaned pigs. J Agric Food Chem 58:7323–7328
16. Shukla R, Cheryan M (2001) Zein: The industrial protein from corn. Ind Crop Prod 13:171–192
17. Tschiersch C, Nikfardjam MP, Schmidt O, Schwack W (2010) Degree of hydrolysis of some vegetable proteins used as fining agents and its influence on polyphenol removal from red wine. Eur Food Res Technol 231:65–74
18. Azarmi S, Roa W, Lobenberg R (2007) Current perspectives in dissolution testing of conventional and novel dosage forms. Int J Pharm 328:12–21
19. Herman R, Gao Y, Storer N (2006) Acid-induced unfolding kinetics in simulated gastric digestion of proteins. Regul Toxicol Pharmacol 46:93–99
20. Kazanov MD, Igarashi Y, Eroshkin AM, Cieplak P, Ratnikov B, Zhang Y, Li Z, Godzik A, Osterman AL, Smith JW (2011) Structural determinants of limited proteolysis. J Proteome Res 10:3642–3651
21. Zhang B, Luo Y, Wang Q (2011) Effect of acid and base treatments on structural, rheological, and antioxidant properties of alpha-zein. Food Chem 124:210–220
22. Cabrera-Chávez F, Ezquerra-Brauer M, Herrera-Urbina R, Rosell C, Rouzaud-Sández O (2010) Physicochemical properties of wheat gluten proteins modified by protease from sierra (*Scomberomorus sierra*) fish. Int J Food Prop 13:1187–1198
23. Bergamo P, Maurano F, Mazzarella G, Iaquinto G, Vocca I, Rivelli AR, De Falco E, Gianfrani C, Rossi M (2011) Immunological evaluation of the alcohol-soluble protein fraction from gluten-free grains in relation to celiac disease. Mol Nutr Food Res 55:1–5

24. Geraghty DE, Messing J, Rubenstein I (1982) Sequence analysis and comparison of cDNAs of the zein multigene family. EMBO J 1:1329–1335
25. van der Merwe PA, Davis SJ (2003) Molecular interactions mediating T cell antigen recognition. Annu Rev Immunol 21:659–684
26. Qiao SW, Sollid LM, Blumberg RS (2009) Antigen presentation in celiac disease. Curr Opin Immunol 21:111–117
27. Freeman HJ, Chopra A, Clandinin MT, Thomson AB (2011) Recent advances in celiac disease. World J Gastroenterol 17:2259–2272
28. Skerritt JH, Devery JM, Penttila IA, LaBrooy JT (1991) Cellular and humoral responses in coeliac disease. 2. Protein extracts from different cereals. Clin Chim Acta 204:109–122
29. Shan L, Molberg O, Parrot I, Hausch F, Filiz F, Gray GM, Sollid LM, Khosla C (2002) Structural basis for gluten intolerance in celiac sprue. Science 297:2275–2279
30. van de Wal Y, Kooy YMC, van Veelen P, Vader W, August SA, Drijfhout JW, Peña SA, Koning F (1999) Glutenin is involved in the gluten driven mucosal T cell response. Eur J Immunol 29:3133–3139
31. Tollefsen S, Arentz-Hansen H, Fleckenstein B, Molberg O, Ráki M, Kwok WW, Jung G, Lundin KE, Sollid LM (2006) HLA-DQ2 and -DQ8 signatures of gluten T cell epitopes in celiac disease. J Clin Invest 16:2226–2236
32. De Vincenzi M, Silano M, Luchetti R, Carratù B, Boniglia C, Pogna NE (1999) Agglutinating activity of alcohol-soluble proteins from quinoa seed flour in celiac disease. Plant Foods Hum Nutr 54:93–100
33. Ciacci C, Cirillo M, Cavallaro R, Mazzacca G (2002) Long-term follow-up of celiac adults on a gluten-free diet: Prevalence and correlates of intestinal damage. Digestion 66:178–185
34. Carroccio A, Ambrosiano G, Di Prima L, Pirrone G, Iacono G, Florena AM, Porcasi R, Noto D, Fayer F, Soresi M, Geraci G, Sciumè C, Di Fede G (2008) Clinical symptoms in celiac patients on a gluten-free diet. Scand J Gastroenterol 43:1315–1321