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Sorghum, a Healthy and Gluten-free Food for Celiac Patients As Demonstrated by Genome, Biochemical, and Immunochemical Analyses

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ABSTRACT: Wheat (*Triticum* spp. L.), rye (*Secale cereal* L.), and barley (*Hordeum vulgare* L.) seeds contain peptides toxic to celiac patients. Maize (*Zea mays* L.) and rice (*Oryza sativa* L.) are distant relatives of wheat as well as sorghum (*Sorghum bicolor* (L.) Moench) and are known to be safe for celiacs. Both immunochemical studies and in vitro and in vivo challenge of wheat-free sorghum food products support this conclusion, although molecular evidence is missing. The goal of the present study was to provide biochemical and genetic evidence that sorghum is safe for celiac patients. In silico analysis of the recently published sorghum genome predicts that sorghum does not contain peptides that are toxic for celiac patients. Aqueous/alcohol-soluble prolamins (kafirins) from different sorghum varieties, including pure lines and hybrids, were evaluated by SDS-PAGE and HPLC analyses as well as an established enzyme-linked immunosorbent assay (ELISA) based on the R5 antibody. These analyses provide molecular evidence for the absence of toxic gliadin-like peptides in sorghum, confirming that sorghum can be definitively considered safe for consumption by people with celiac disease.

KEYWORDS: *sorghum cultivars, gliadins, kafirins, SDS-PAGE, HPLC, ELISA, bioinformatic analysis*

INTRODUCTION

Celiac disease (CD) is a chronic inflammatory disease of the small intestine caused by the ingestion of proline- and glutamine-rich wheat gluten (consisting of the gliadin and glutenin subcomponents) or related proteins from rye and barley flours.[1] The activation of gluten-reactive T cells represents a key event in disease development. Gluten-reactive T cells from celiac lesions predominately recognize gluten peptides in which glutamine residues at certain positions have been converted to glutamic acid by tissue transglutaminase-mediated deamination.[2,3] Several different celiac T cell epitopes derived from gliadin proteins have been identified.[2,4−7] These epitopes cluster in the proline-rich regions of the proteins.[5] The epitopes identified in α-gliadins appear to be particularly important because they are recognized by intestinal T cells of the majority of adult celiac patients [2]

In vivo (instillation) testing established the toxicity of α-type gliadins, and in vitro (organ culture) testing of gliadin peptides demonstrated that the N-terminal region (domain I) of α-gliadins is involved in activating CD. The longest sequences common for toxic peptides were found to be −PSQQ− and −QQQP−. Various in vitro tests and two in vivo studies on synthetic peptides support the importance of one or both of these sequences. Equivalent protein fractions of rye, barley, and probably oats were also considered to be toxic.[8,9] More recently, by mimicking the enzymatic gastrointestinal digestion of a representative α-gliadin (α_2-gliadin), Shan and co-workers[10] found a 33-mer (LQLQPFPQPQLPYPQPQL-PYPQPQLPYPQPQPF; α_2-gliadin 56−88) to be particularly interesting. This fragment was resistant to further breakdown by luminal proteases and intestinal brush-border enzymes due to its high proline content (13 of 33 residues are proline), and the deamidated 33-mer was found to be an extremely potent T cell stimulator, several-fold more potent that any other known gluten peptide.[10] Other studies demonstrated that a 33-mer of

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Table 1. *Sorghum bicolor* (L.) Moench Cultivars and Their Origins

genotype code	variety name	originator	specific character
Macia	Macia	ICRISAT	food-grade, malt-sorghum variety
05MN5113	86EO361	Texas A&M University	food-grade sorghum
05MN5115	91BE7414	Texas A&M University	food-grade sorghum
Tx436	Tx436	Texas A&M University	food-grade sorghum parent line
Fontanelle1000	F1000	NU Market	food-grade sorghum
Epuripur	2K x 17/B/1	Nasarri, Uganda	food-grade sorghum
Sekodo	E525Ht.Red	Nasarri, Uganda	food-grade sorghum

α-gliadin that is the end product of normal gastrointestinal proteolysis can be presented to T cells without further processing by antigen-presenting cells.[11]

People genetically predisposed to CD suffer an immune reaction to gluten proteins found in all *Triticum* species and closely related cereals such as barley and rye.[12] The only treatment for CD is lifelong avoidance of gluten proteins. Sorghum is a drought- and heat-tolerant cereal grain that grows in semiarid conditions. Whereas sorghum has traditionally been used primarily as animal feed in Western countries, nearly 40% of the world sorghum production is used for human food in Africa and India.[13,14] Sorghum is considered a safe food for celiac patients, because it is more closely related to maize than to wheat, rye, and barley.[12,15] In recent years, farmers in the United States have begun producing sorghum hybrids that produce white grain from a tan-color plant (often called "food-grade" sorghum) for production of wheat-free foods for persons with CD.[16] Moreover, new technologies aimed at enhancing the nutritional and functional values of sorghum proteins in industrial-scale processes have been developed.[17] Therefore, sorghum might provide a good basis for gluten-free foods for people in other parts of the world and specifically for celiac patients. Both immunochemical studies[18−20] and in vitro and in vivo challenges of wheat-free sorghum food products[21] support this conclusion.

Given the growing importance of food-grade sorghum for human consumption,[21,22] more studies are needed to support the cultivation and consumption of these varieties in new environments such as Mediterranean countries.[23−25] The aim of the present study was to provide molecular evidence that sorghum does not contain grain proteins that may be toxic for celiac patients providing genetic evidence by an analysis of the sorghum genome and by examining biochemical characteristics of more and different white grain sorghum varieties.

MATERIALS AND METHODS

Plant Cultivars. The plant cultivars and source employed in this study are indicated in Table 1.

Field trials were conducted in southern Italy at the CRA-Centre for Cereal Research of Foggia (41° 28′ N, 15° 32′ E, and 75 m asl) on a clay-loam soil (Typic Chromox-erert) during 2010. The sorghum varieties were evaluated in 2010 during the spring/summer period, whereas the durum wheat (cv. Svevo), used as wheat control, was cultivated during the 2009−2010 growing season in an adjoining field. The two species have different thermal requirements (macrotherm vs microtherm for sorghum and wheat, respectively), so sorghum genotypes were sowed when wheat was harvested in the entire region, not just in the adjoining field. In addition, in the field there was no risk of contamination because the equipment used for harvesting grains was different. In the laboratory, to avoid cross-contamination, all of the necessary precautions were adopted.

The experimental design was a randomized complete block replicated three times with plots of 45 m size. Individual plots were rows wide. Row length was 1.5 m. The test plots were planted and harvested with equipment designed for small-plot work. The sowing date was May 10, 2010, and the plots were harvested on October 7. Fertilizer applications were made at the rate of 1/3 before sowing (incorporated by disk harrowing) as ammonium phosphate, and 2/3 N top-dressed applied at the tillering stage as ammonium nitrate. Weeds within the growing season were controlled by means of specific herbicides.

Flour Sample Preparation. A 500 g sample of dried grain was milled to obtain a fine flour by means of a Chopin CD1 laboratory mill (Tripette and Renaud group, Villeneuve-la-Garenne, France) and a planetary sieve (Buhler, Uzwil, Switzerland) through a 120 μm^2 sieve opening.

Protein Extraction. Separation of alcohol-soluble protein was carried out as previously described.[26] Pre-extraction of albumins and globulins from non-defatted flour (100 mg) was achieved by stepwise addition (three times) of 1 mL of 0.4 M NaCl, 0.067 M $HKNaPO_4$, pH 4.6. Subsequently, proteins were extracted with 0.5 mL of 70% v/v ethanol (three times). Each extraction step started with vortexing for 2 min at room temperature and magnetic stirring for 10 min. The suspension was centrifuged for 20 min at 7000 rpm using a refrigerated centrifuge. The supernatants of alcohol-soluble proteins were combined, and aliquots of each extract were stored at −20 °C and then used for biochemical and immunochemical analyses. Stability of the samples was checked by SDS-PAGE.

HPLC Analysis. Twenty-five microliter aliquots were filtered through a 0.45 μm polyethersulfone (PES) membrane (Millipore, Milan, Italy) and analyzed by RP-HPLC using a C8 (Vydac 208TP52, 5 μm, 2.1 mm × 250 mm) reversed-phase column with a flow rate of 0.2 mL/min on a Hewlett-Packard (Waldbronn, Germany) model 1100 system with an integrated diode array detector. The column temperature was 55 °C, and the eluent was monitored at 210 nm. Solvent A was 0.1% trifluoroacetic acid (TFA) in water, and solvent B was 0.1% TFA in acetonitrile. The column was equilibrated at 25% solvent B. Separation of the peptides was performed with a gradient of 25−55% solvent B over 100 min. The detection limit was 48 ppm gliadins.

SDS-PAGE Analysis. Twenty-five microliter aliquots were lyophilized and dissolved in 20 μL of buffer (0.125 M Tris-HCl pH 6.8, 5% SDS, 20% glycerol, 5% (w/v) β-mercaptoethanol, 0.02% bromophenol blue containing 4 M urea and 0.1% dodecylmaltoside solution) and boiled for 5 min. The sample was loaded onto 12% polyacrylamide gel. Electrophoresis was carried out using the Tris−glycine−SDS buffer system (25 mM Tris, 192 mM glycine, and 0.1% SDS) on a Hoefer SE 600 apparatus (Amersham) at 30 mA/gel until the dye front reached the bottom edge of the gel. Broad range molecular weight standard (ranging from 200 to 7 kDa) (Bio-Rad Laboratories, Hercules, CA, USA) was used to calibrate the gel. Gel was fixed and stained with Coomassie Brilliant Blue G-250. The detection limit was 72 ppm gliadins.

Preparation of Gliadin Standard. A wheat gliadin standard was prepared from durum wheat (cv. Svevo) using the cocktail extraction kit (article R7006, R-Biopharm AG, Darmstadt, Germany) as previously described.[27,28] In brief, each 0.5 g dried flour sample was placed into a 12 mL sterile polypropylene tube. A total of 5 mL of the extraction cocktail was added to each tube containing the sample. The tubes were closed tightly and the caps covered with Parafilm to avoid evaporation. The mixtures were mixed thoroughly by vortexing (5−10 s), and the tubes were placed in a rack. The tubes were incubated in an

oven at 50 °C for 40 min. The tubes were allowed to cool for 5 min at room temperature. The tubes were opened, 7.5 mL of 80% ethanol was added, and the samples were dispersed thoroughly by vortexing (10−60 s) until total dispersion of the sample; the tubes were then incubated for 1 h at room temperature in a rotary shaker at 45 turns/min. The tubes were centrifuged at 2500*g* for 10 min at room temperature. One milliliter of each supernatant was transferred with a micropipet into clean sterile 2 mL Eppendorf tubes and centrifuged for 5 min at 2500*g* in an Eppendorf centrifuge at room temperature. The supernatants were transferred using sterile Pasteur pipets to clean sterile Eppendorf tubes. A commercial gliadin standard from wheat (Sigma G-3375, St. Louis, MO, USA) was also used.

Sandwich Enzyme-Linked Immunosorbent Assay. The RIDASCREEN standard test kit [RIDASCREEN Gliadin (article R7001), R-Biopharm AG] sandwich ELISA based method was used to identify gliadins in grain flour samples according to both Valdés et al.[27] and the manufacturer's instructions. Detection and quantification limits were 1.5 and 2.5 ppm gliadins, respectively. Determinations were carried out according to the instruction leaflets sent with the kit. The concentrated solution of conjugate, sample dilution buffer, and washing buffer supplied with the kit were diluted as indicated in the R-Biopharm AG protocol. The measurements were made photometrically at 450 nm. The absorbance was proportional to the gliadin concentration of the sample in comparison to the calibration curve with gliadin standards.[29] The measurements were made on three separate ELISA extractions, on the same day, in parallel experiments.

In Silico Analysis. The nucleotide sequences of kafirin genes from the *Sorghum bicolor* (L.) Moench genome[30] were available at the site http://pgir.rutgers.edu/. The nucleotide sequences were translated into amino acid sequences by the software GeneJockey Sequence Processor (published and distributed by Biosoft) and searched for the presence of toxic gliadin-like tetrapeptides −PSQQ− and −QQQP− by using the same software. DNA similarity searches were carried out using BLAST at NCBI (http://www.ncbi.nlm.nih.gov/). Sequence alignments were performed with Clustal W at EBI (http://www.ebi.ac.uk/).

Statistical Analysis. Samples were extracted and analyzed in triplicate, and statistical differences were evaluated using Kruskal−Wallis nonparametric ANOVA and Statistica software (StatSoft version 7.1, StatSoft, Inc., Tulsa, OK, USA) with a P value of <0.05.

RESULTS

In Silico Genome Analysis. The Bioinformatic database represents a major tool to acquire information concerning the bioactive proteins, including information about the source of origin, the structure of their epitopes, and potential biological protein activity.

The recently published nucleotide sequences of sorghum kafirin genes[29] (http://pgir.rutgers.edu/) were translated and probed for the existence of putative toxic gliadin-like peptides using the tetrapeptides −PSQQ− and −QQQP− (see Introduction) as bait. Alignment of deduced amino acid sequences of sorghum kafirins is shown in Figure 1. The analysis demonstrated that although kafirins exhibit a high content of glutamines often arranged in long runs, these runs never ended in proline, and the above-mentioned toxic tetrapeptides as well as the 33-mer (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) were absent in the sorghum storage proteins.

This result supports a report by Ciacci et al.[21] in which no differences were observed in the number of positive cells for the inflammatory markers examined among biopsies cultured with or without sorghum.

As a further confirmation, a characterization of different cultivars including pure lines and hybrids was done to explore the genetic protein variability. The most widely used methods

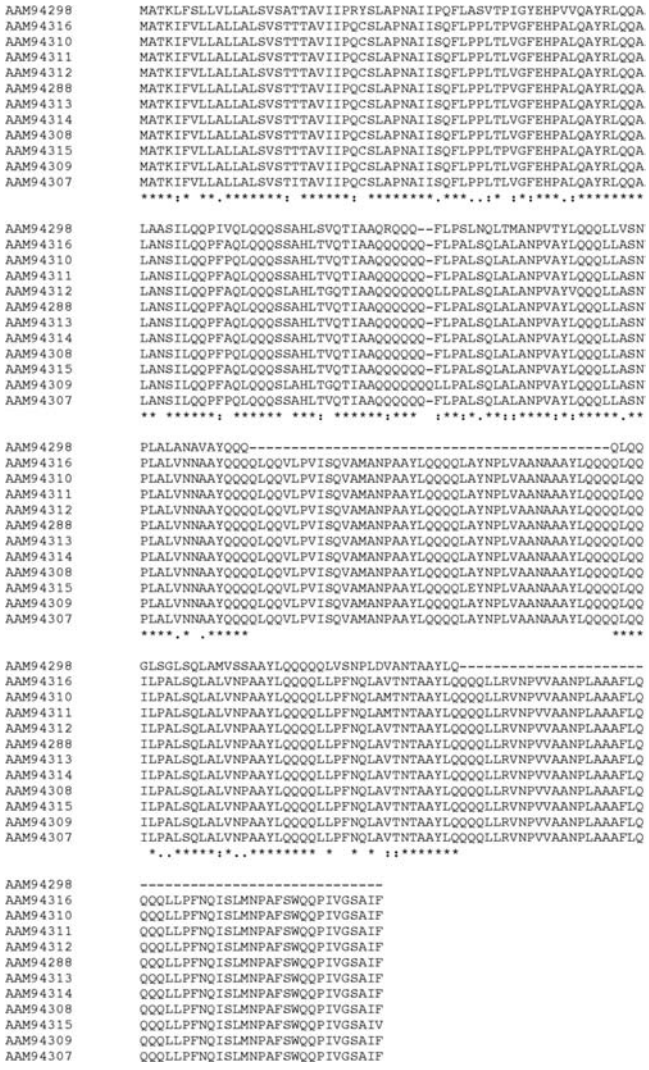

Figure 1. Clustal W alignment of kafirins from *Sorghum bicolor* (L.) Moench genome. Translated kafirin genes are annotated at the site http://pgir.rutgers.edu/ with the following protein accession numbers: AAM94307, AAM94308, AAM94309, AAM94310, AAM94311, AAM94312, AAM94313, AAM94314, AAM94315, AAM94316, AAM94288, and AAM94298. (∗) Identical residue; (:) conserved substitution; (.) semiconserved substitution. In all sequences are absent −PSQQ− and −QQQP− motifs.

to separate cereal proteins are sodium dodecyl sulfate (SDS)−PAGE and reversed-phase (RP)-HPLC.

Biochemical Evidence for the Absence of Gliadin-like Peptides in Sorghum Varieties. Alcohol-soluble protein profiles (kafirins) from seven cultivars of sorghum were compared to the gliadin profile from a common Italian wheat variety, Svevo, using SDS-PAGE and RP-HPLC analysis.

To obtain molecular weight information, the alcohol-soluble proteins were analyzed by SDS-PAGE (Figure 2). A consistent number of bands sizing between 31 and 66 kDa were detected for gliadin analysis in the wheat cultivar. In particular, the most abundant α-kafirins (the most abundant, 80−85% of total kafirin) presented two bands at 23 and 25 kDa, followed by the β-kafirins (7−13%) at 19 kDa, and finally γ-kafirins (10−20%) at 20 kDa, in agreement with Belton et al.[31] Each protein class is further divided into subclasses. Whereas SDS-PAGE has been useful in studying kafirin proteins, it has low resolution in separating the kafirin subclasses. The RP-HPLC method has

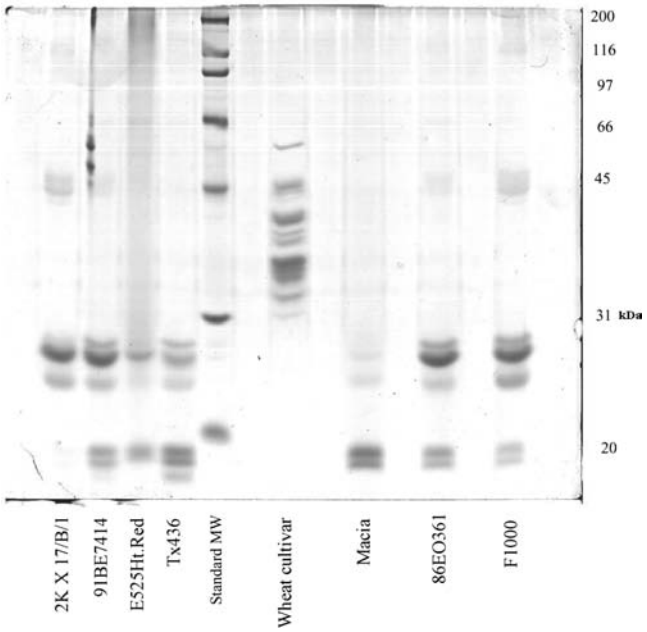

Figure 2. SDS-PAGE of kafirins extracted from sorghum varieties (2Kx17/B/1, 91BE7414, E525Ht.Red, Tx436, Macia, 86EO361, F1000) compared to gliadin extracted from wheat variety Svevo.

been used to separate kafirin subclasses with improved resolution on the basis of surface hydrophobicity.[26]

The HPLC chromatogram of gliadin (Figure 3a) showed a heterogeneous protein profile that could be grouped, on the basis of retention time, in ω-, α/β, and γ-gliadins, as previously reported.[21,32] On the contrary, completely different UV chromatograms were observed with kafirins (Figure 3b–h). In all sorghum varieties, UV chromatograms showed signals with retention time of>90 min, indicating that kafirins were more hydrophobic than gliadins. Differences in the retention time and/or number of peaks were also present among the sorghum varieties. Varieties Tx436 and 91BE7414 (Figure 3, panels b and c, respectively) were quite similar as well as variety E525Ht.Red with respect to variety 86EO361 (Figure 3, panels g and h, respectively) and variety 2Kx17/B/1 with respect to F1000 (Figure 3 panels e and f), respectively). A simpler profile was observed for variety Macia (Figure 3d), the chromatogram of which exhibited a single predominant peak.

In conclusion, both HPLC and SDS-PAGE data provided evidence that proteins with characteristics similar to those of wheat gliadins are absent in all sorghum varieties analyzed.

Immunochemical Evidence for the Absence of Gliadin-like Peptides in Sorghum Varieties. ELISAs of kafirns from different sorghum varieties were performed to confirm the absence of gliadin-like peptides in sorghum by a more sensitive method. Table 2 shows the results of immunochemical measurement of gliadin concentration in sorghum flour samples produced by milling of different sorghum grain cultivars. The results indicated that the gluten levels in sorghum flours were <5 ppm. These values are well below the 20 ppm threshold that has been proposed to be safe for celiac patients.[27]

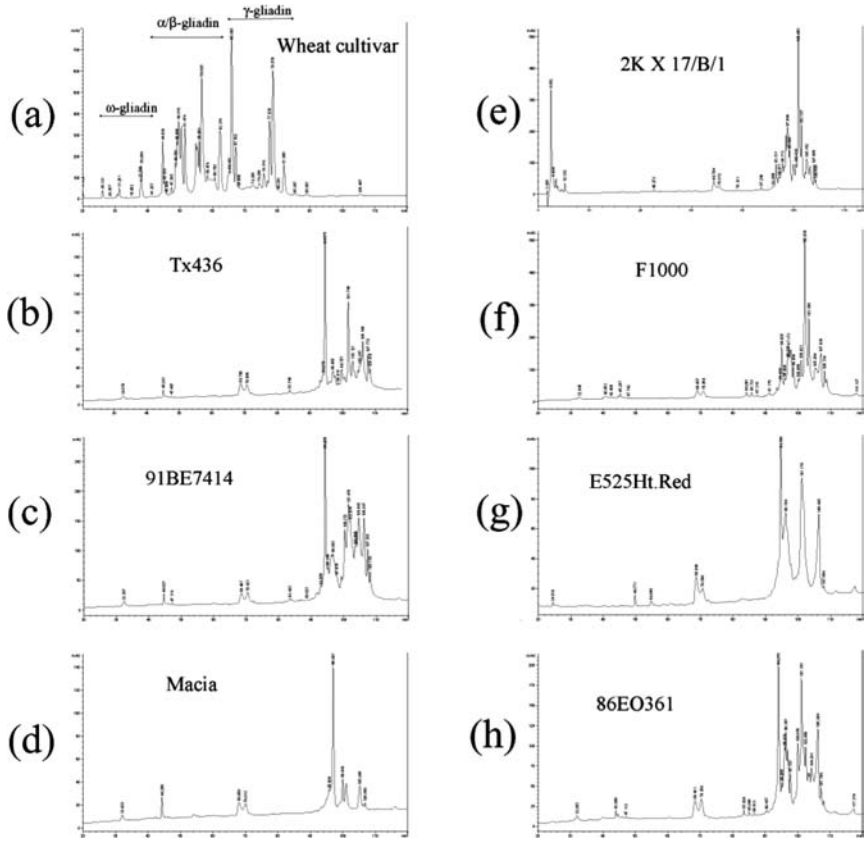

Figure 3. RP-HPLC chromatograms of kafirins extracted from sorghum varieties (panels b–h) compared to gliadin extracted from wheat variety Svevo (panel a).

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Table 2. Measurement of Gliadin in Flours by Using Sandwich R5 Enzyme-Linked Immunosorbent Assay (ELISA)

strain	type	content[a] (ppm)
Macia	sorghum	<5
86EO361	sorghum	<5
91BE7414	sorghum	<5
Tx436	sorghum	<5
F1000	sorghum	<5
Epuripur	sorghum	<5
Sekodo	sorghum	<5
gliadin standard[b]	wheat	56

[a]Mean values from three measurements. [b]Gliadin standard from wheat (Sigma).

DISCUSSION

In this study in silico genomic approaches and biochemical experiments were used to characterize protein profiles of storage proteins in different sorghum varieties. These analyses provide molecular evidence for the absence of toxic gliadin-like peptides in sorghum. This finding was supported by immunochemical experiments using the monoclonal antibody R5, which reacts with the gliadin fractions from wheat and corresponding prolamins from rye and barley. It recognizes among others the toxic sequence QQPFP, which occurs repeatedly in the prolamin molecules. There were no cross-reactions with soy, oats, corn (maize), rice, millet, teff, buckwheat, quinoa, and amaranth. This antibody is recommended by Codex Alimentarius[33,34] for use in the sandwich ELISA method for determination of gluten content in gluten-free food.

Thus, this cereal, similarly to maize and rice, might be considered safe for gluten-intolerant people. Moreover, sorghum is an inexpensive grain, and therefore it should be possible to produce sorghum-derived food products at low cost. The flour produced from white sorghum hybrids is light in color and has a bland, neutral taste that does not impart unusual colors or flavors to food products.[21] Furthermore, from a nutritional point of view, sorghum flour is characterized by high total lipid levels, in particular linoleic acid (the predominant) and oleic acid. Unsaturated fatty acids are of great importance to their antiaggregating activity on blood lipoprotein particles. All of these attributes make it desirable for use in wheat-free food products.

Although our results provide molecular evidence that celiac patients may consume sorghum safely, there is a concern about the possibility of using it as a food source because of condensed tannins that reduce the digestibility of dietary proteins. However, the modern, food-grade sorghum cultivars described in this paper do not contain condensed tannins and were developed for use as ingredients in food products for human consumption.[14] Food-grade sorghum cultivars incorporate several key traits including white pericarp color, thin mesocarp, normal endosperm type, no condensed tannins in the testa, and tan-plant necrotic lesion color.[16] The tannin content of sorghum is controlled by two major genes with additional modifiers, and modern sorghum cultivars contain mutations in one or both genes.[35] The nutritional qualities of food-grade sorghum are excellent.[36] Food-grade sorghums should be considered as an important option for all people, especially celiac patients.

Another interesting point is raised by comparison of UV chromatograms of kafirins from the different sorghum varieties showing significant differences in the retention time and/or number of peaks in the different cultivars. Varieties Tx436 and 91BE7414, E525Ht.Red and 86EO361, and 2Kx17/B/1 and F1000 could be double-clustered, respectively, whereas a simpler profile was observed for the variety Macia, a food-grade sorghum variety also used for malt production, the chomatogram of which exhibited a single predominant peak. Currently, kafirins are classified into α-, β-, and γ-kafirins on the basis of differences in solubility, molecular weight, structure, and their relationships to the zeins revealed by their amino acid compositions, sequences, and immunochemical cross-reactions.[31,37−40] This classification is similar to the nomenclature system of the zein of maize proposed by Esen.[41] A fourth group, related to the δ-zeins of maize, has been identified from the sequences of cloned DNAs but has not been characterized at the protein level.[31] The α-kafirins comprise about 80−84% of the total kafirin in vitreous endosperm[42] and 66−71% in opaque endosperm[43] and are composed of two polypeptides with molecular weights of 25 and 23 kDa.[38] Recent studies have shown that α-kafirins can be divided into two classes related most closely to the M_r 19 and 22 kDa α-zeins, respectively.[31,44] The study of kafirin profiles in different sorghum varieties may shed light on the evolution of modern sorghum because these are some of the most relevant domestication traits. Indeed, kafirins are the major seed storage proteins in sorghum and also have great economical importance because they represent a major biological nitrogen source and a renewable source of essential amino acids for both human and animal diets. Therefore, a variation in the kafirin profiles may have had an impact on the nutritional value and promoted the selection of sorghum cultivars in different geographical areas.

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Notes

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