The European Journal of Plant Science and Biotechnology ©2012 Global Science Books

Western Blot Analysis Uncovers Clues to Prolamin Digestibility in Raw and Cooked Meal from Sorghum and Corn

Joshua H. Wong[1] • Jeffrey F. Pedersen[2] • Bob B. Buchanan[1] • Peggy G. Lemaux[1*]

[1] Department of Plant and Microbial Biology, University of California, Berkeley CA 94720 USA
[2] United States Departments of Agriculture, Agricultural Research Service, Lincoln NE 68583 USA

Corresponding author: * **lemauxpg@berkeley.edu**

ABSTRACT

Digestibility of cereal grain protein is conventionally expressed as percentage of protein digested by pepsin in a defined period. Sorghum has the lowest protein digestibility among cereals especially after wet cooking. If additional details were known as to which proteins are resistant to digestion, it should be possible to find means to improve this property. In this paper, two approaches are described to extract and identify proteins undigested by pepsin in uncooked and cooked meal from numerous sorghum and corn varieties. The first, one-solvent approach involves extracting residues undigested after 2 h with Borate-SDS-ME and separating by PAGE. Improved separation of undigested sorghum proteins was achieved using NuPAGE Bis-Tris gels. Western blots with antibodies against particular zeins and kafirins, used to monitor fates of different kafirins, revealed differential digestion rates. A second approach involved extracting undigested residues sequentially with 60% *t*-butanol, 60% *t*-butanol-ME and Borate-SDS-ME. The second, three-solvent approach, coupled with western analysis, revealed the following. (i) Oligomeric forms of certain kafirins exist that differ in the degree of their susceptibility to pepsin digestion. (ii) Effect of cooking on the formation and digestion of the oligomers. (iii) Cross-linked forms of most α-kafirins became more resistant to digestion after cooking. (iv) Most α-kafirins are preferentially extracted in 60% *t*-butanol-ME while most γ-kafirins are extracted in Borate-SDS-ME buffer. (v) Monomeric γ-kafirins are resistant to pepsin digestion. (vi) γ-Kafirins form a series of oligomers that exhibit differential resistance to digestion. Our results suggest that the presently described systematic approach to analyzing the digestion by pepsin of sorghum prolamins should lead to greater insights into the digestion of specific types of sorghum grain proteins.

Keywords: disulfide proteins; sorghum; starch-protein interface
Abbreviations: DAB, 3, 3'-diaminobenzidine; **M_r**, relative molecular mass; **ME**, 2-mercaptoethanol; **MOPS**, 3-(*N*-morpholino) propane sulfonic acid; **PAGE**, polyacrylamide gel electrophoresis; **SDS**, sodium dodecyl sulfate; **TMB**, 3, 3', 5, 5' tetramethylbenzidine

INTRODUCTION

Poor protein digestibility in sorghum grain has negative nutritional implications in arid regions of Africa and Asia for millions of the world's poorest people who depend on it as their main staple food. Higher protein digestibility is needed to meet the nutritional needs of these human populations, who have marginal protein intake, as well as those of animals (Shewry 2007). The improvement of sorghum digestibility and nutrition are among the major objectives of sorghum research. It is known that many factors, both endogenous and exogenous, are under the influence of genetics and environment, and are purported to be responsible for sorghum's relatively low protein digestibility (Duodu *et al.* 2003). Recent results from principal component analysis revealed that the content of amylose and total starch, together with protein digestibility, account for 94% of variation in sorghum digestibility (Wong *et al.* 2010). In this paper our focus is specifically on grain protein digestibility.

Kafirin was named as the first major alcohol-soluble protein isolated from kafir, also known as *Andropogon sorghum* (Johns and Brewster 1916), a synonym of *Sorghum bicolor* (L.) [USDA Germplasm Resources Information Network (GRIN)]. This prolamin is the storage protein found in sorghum endosperm that comprises about 70% of total protein in whole grain sorghum flours (Hamaker *et al.* 1995). The remaining protein consists of non-kafirins (*i.e.*, albumins, globulins and glutelins) that are involved in cellular functions (Hamaker and Bugusu 2003). Four subclasses of kafirins, α-(23 and 25 kDa), β-(20 kDa), γ-(28 kDa) and δ-(10 kDa), have been described, based on their protein characteristics and more recently on DNA sequence (Shull *et al.* 1991; Mazhar *et al.* 1993; Belton *et al.* 2006). Each kafirin shares a high degree of homology with the equivalent class of zein proteins from corn (Garratt *et al.* 1993; Watterson *et al.* 1993).

The kafirins are synthesized and translocated into the lumen of the endoplasmic reticulum where they form protein bodies (Taylor *et al.* 1985). The development of protein bodies in sorghum may be similar to the process in corn where detailed studies have been done. In corn, as demonstrated by immunolocalization, developing protein bodies initially form as small accretions of β- and γ-zeins (Lending and Larkins 1989). The α- and δ-zeins subsequently accumulate within this network as discrete locules that expand and aggregate to fill the interior. As a result, the β- and γ-zeins are concentrated around the peripheral layer of the more mature protein body. Interactions among zein proteins are key determining factors in protein body assembly (Kim *et al.* 2002; Coleman *et al.* 2004).

In sorghum, α-kafirins comprise 70-80% of total kafirins (Hamaker *et al.* 1995), followed by the γ- (~ 15%), β- (~ 5%) and δ- (< 1%) kafirins. α-Kafirins have low levels of cysteine residues relative to the β- and γ-types (5 and 7 mol%, respectively), whereas δ-kafirins lack cysteine but are rich in methionine (16-18 mol%). The γ-kafirin is localized at the periphery of the protein body where it may have more of a structural function (Laidlaw *et al.* 2010) than a storage role. Storage proteins of sorghum and corn, kafirins and zeins respectively, have been reported to be relatively more stable to proteases than other prolamins, possibly due to extensive disulfide linkages (Hamaker *et al.*

Received: 1 October, 2011. Accepted: 3 December, 2011.

Original Research Paper

1987). Studies in sorghum indicate that γ-kafirin is fairly resistant to proteolysis due to formation of a disulfide-bond polymer network with itself and/or with β-kafirin (Shull *et al.* 1992; Oria *et al.* 2000), thus protecting the more digestible and centrally located α-kafirins (Oria *et al.* 1995a).

The α-kafirins are more predominant in the outer layers of the grain, comprising 80 to 84% of the total kafirin fraction in vitreous endosperm and 66 to 71% in opaque endosperm (Watterson *et al.* 1993). In the corneous endosperm, the tightly packed protein matrix (consisting of protein bodies and non-kafirins) surrounds the starch granules (Taylor *et al.* 1984; Shull *et al.* 1990; Hamaker and Bugusu 2003) and acts as a barrier to starch gelatinization and digestibility (Chandrashekar and Kirleis 1988; Duodu *et al.* 2002; Ezeogu *et al.* 2005) due to cross-linking between γ- and β-kafirins and matrix proteins (Oria *et al.* 1995a; Duodu *et al.* 2001; Hamaker and Bugusu 2003).

Cooking reduces protein digestibility by promoting formation and interchange of disulfide linkages (Axtell *et al.* 1981; Hamaker *et al.* 1987; Oria *et al.* 1995b; Duodu *et al.* 2001, 2002). The negative impact of cooking on protein digestibility has been mitigated by addition of 2-mercaptoethanol (ME) or other reducing agents (Hamaker *et al.* 1987; Elkhalifa *et al.* 1999). These observations have strengthened the concept that disulfide bond formation plays a critical role in reducing sorghum protein digestibility, and this is widely accepted as a mechanism that acts to reduce sorghum protein digestibility.

Nitrogen (N) content is conventionally measured by combustion and protein content, calculated as N × 6.25 (Mosse 1990). Protein digestibility following treatment is expressed as the proportion of remaining protein divided by initial protein. But gross protein digestibility does not identify which protein or proteins are poorly digested and remain in the residue. Better knowledge of the nature of these undigested proteins might provide a means to devise solutions to the problem. In 2001, Aboubacar *et al.* (2001) published a rapid gel-based procedure to analyze proteins remaining in the pepsin-undigested residue in a time-dependent manner. Digestibility of individual proteins in a highly digestible mutant line of sorghum with high lysine content was compared to a wild-type variety of sorghum known to be less digestible. Their conclusion was that α-kafirin was the main protein remaining undigested by pepsin after a 2 h treatment of meal from the highly digestible sorghum line. Since then, results of several studies have been published using similar procedures to dissect sorghum protein digestibility (Nunes *et al.* 2004; Wong *et al.* 2009, 2010).

We have adapted a similar pepsin digestion protocol using a novel system of NuPAGE SDS gels that resulted in improved separation of the extracted proteins and excellent resolution of the different kafirin types. This procedure also provides for comparison of protein digestion rates among different sorghum lines (Wong *et al.* 2009, 2010). However, it is important to note that the migration patterns of the different kafirins are slightly different from those seen with the traditional Tris-Glycine gel system. An account of the results using a gel-based system with a one-solvent extraction method has been reported (Wong *et al.* 2010). In the present paper we present a detailed and systematic approach to describe the fate of various kafirins during *in vitro* pepsin digestion of uncooked and cooked sorghum meal from selected sorghum lines that differ in their reported digestibility using one- and three-solvent extraction protocols. Western blots employing various anti-zein and -kafirin antibodies were used to identify and follow the oligomeric and monomeric forms of the different kafirin types.

MATERIALS AND METHODS

Materials

Several sorghum lines were used. 'Tx7078', 'IA19', 'RTx430' were obtained from USDA-ARS, Lincoln, NE; 'P898012' was obtained from Pioneer Hi-Bred (Johnston IA); '296B' was obtained from Dr. Ian Godwin, University of Queensland, Australia; 'TCD898012' ('TCD') is a line derived from *in vitro* tissue culture of 'P898012'. Corn line 'B73' and its near-isogenic waxy (low amylose) counterpart 'T6-9WX' were obtained from Dr. Jay Hollick, UC Berkeley. Some of these seeds' characteristics were described previously (Wong *et al.* 2010). In brief, all sorghum lines are non-waxy; 'IA19', 'P898012' and 'TCD' seeds have a tannin-containing sub-coat. *In vitro* protein digestibility ranked as follows: 'T6-9WX' > 'B73' > 'P898012' > '296B' > 'IA19' > 'Tx7078' > 'RTx430' (71.5%, 70.6%, 58.9%, 52.7%, 44.8%, 42.4%, 42.1%, respectively; SE = 2.6) (Wong *et al.* 2010). Protein digestibility of 'TCD' was not previously described.

Pepsin (porcine stomach mucosa, P-7000) was from Sigma-Aldrich (St. Louis, MO). NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA) or the equivalent Criterion XT Bis-Tris gels (Bio-Rad, Hercules, CA) were used for protein separation using MOPS buffer, pH 7.7 (Invitrogen). Two protein molecular weight standards were used: SeeBlue® Plus2 Prestained Standard (Invitrogen) and PageRuler Plus Prestained Protein Ladder (Fermentas Inc., Hanover, MD).

Mono-specific antibodies against 19 kDa α-zein (B1), 22 kDa α-zein (B), 27 kDa γ-zein, 50 kDa γ-zein and 10 kDa δ-zein were from Dr. Rudolf Jung, Pioneer Hi-Bred. Antibody against α-, β- and γ-kafirins was obtained from Dr. Bruce R. Hamaker (Purdue University, West Lafayette, IN).

Methods

1. Preparation of meal

Samples of mature dried sorghum and corn grain were ground in a Wiley Mill through a 40-mesh screen. Ground meal was stored in sealed conical tubes for short-term use at ambient temperature and for long-term use at 4°C. Each sample was composed of two subsamples. Three replications were conducted for protein digestibility with one solvent extraction, and 2 replications with three-solvent extraction.

2. Cooking of sorghum and corn meal

Five hundred mg of meal was mixed with 5 parts of ddH_2O (distilled deionized H_2O) in 50-mL Falcon tubes and placed in a boiling water bath for 20 min with occasional mixing. The gruel was freeze-dried and then re-ground with a mortar and pestle. The fine-powdered meal was stored in 50-mL tubes at ambient temperature and used as cooked samples.

3. Protein content

Nitrogen concentration, N × 6.25 (Mosse 1990), of sorghum samples (3 × 5-6 mg of meal) was quantified by Micro-Dumas combustion method (http://www.uga.edu/sisbl/udumas.html) using CE Instruments NC-2100 elemental analyzer (Thermo Fischer Scientific, Inc., Waltham, MA). Acetanilide was used as standard.

4. Protein digestibility

Total protein of control and residual protein of 2 h pepsin-digested samples were quantified as described above. Protein digestibility was calculated as the difference between total protein and residual protein after 2 h of pepsin digestion, divided by the total protein; results were expressed as a percentage.

5. Gel-based assay of time course of in vitro pepsin digestion with one-solvent extraction

In vitro pepsin digestion at 37°C for various times, extraction of pepsin-undigested protein using Borate-SDS-ME buffer, pH 10 and their subsequent separation on Bis-Tris gels with MOPS buffer, pH 7.7 were as described (Wong *et al.* 2009, 2010) (**Fig. 1**, path A). When proteins were separated under non-reducing conditions, 2-ME was omitted from NuPAGE Sample Buffer in preparing samples.

Table 1 Relative percent of high vs. low M_r regions and percentage of each region after subjecting to 2 h pepsin digestion.

Line	% high M_r to low M_r regions in untreated control lanes*	% protein digested in high M_r region#	% protein digested in low M_r region#
'T6-9WX'	59.5 to 39.5	67.5	61.2
'B73'	60.0 to 40.0	70.1	61.0
'RTx 7078'	52.0 to 48.0	87.3	54.4
'IA19'	54.6 to 45.4	82.5	41.9
'P89012'	57.6 to 42.4	60.1	38.9
'TCD'	57.5 to 42.5	60.8	52.2
'Tx430'	56.2 to 43.8	81.5	45.9
'296B'	57.7 to 42.3	85.7	44.0

* Relative percent calculated as adjusted volume (intensity x area) of HM_r to LM_r regions in untreated control lanes.
\# % Protein digested in each region = (control minus 2 h pepsin digestion)/control × 100

6. Gel-based assay of in vitro pepsin digestion with three-solvent extraction

Pepsin digestion was as above except 2 h only and pepsin-undigested residues were sequentially extracted with 0.5 mL each of (i) 60% *t*-butanol, (ii) 60% *t*-butanol with 2-ME and (iii) 0.0125M Borate-2%SDS-1% ME buffer, pH 10 (**Fig. 1**, path B). Extraction was for 1 h with continuous shaking at ambient temperature. Extracts were recovered by centrifugation and used for SDS-PAGE and western analysis after protein determination by the Noninterference Protein Assay.

7. NuPAGE analysis of three-solvent extracted kafirins and glutelins

Extracted protein samples after *in vitro* pepsin digestion were analyzed by NuPAGE under non-reducing conditions. Novex Bis-Tris gels (Invitrogen) or Criterion XT Bis-Tris gels (Bio-Rad) provided better separation and resolution of small- to medium-sized proteins due to the neutral pH environment that minimizes protein modifications (Bean 2003; Wong *et al.* 2009, 2010). Aliquots (usually 100 µL) of protein extract were precipitated with 5 vol of acetone at -20°C overnight (o/n). Pellets recovered after centrifugation were re-dissolved in 50 µL 1X NuPAGE sample buffer with no 2-ME, boiled for 5 min, clarified by quick spin and then run at RT on a 12% Bis-Tris gel with MOPS buffer, pH 7.7, for 1 h 20 min at constant voltage of 150 V. M_r standard used was SeeBlue® Plus2 Prestained Standard (Invitrogen). Gels were stained with colloidal Coomassie G-250 o/n and de-stained in several changes of ddH_2O.

8. Non-interference (NI) protein assay

This assay was used to determine concentrations of extracted protein. Interfering substances were removed by precipitating protein using Universal Protein Precipitation Agent (UPPAI and UPPAII), according to manufacturer's instructions (GBiosciences, St. Louis, MO). Amount of copper ion not binding to the peptide backbone of the solubilized protein was determined colorimetrically at 480 nm; bovine serum albumin (BSA) was used as standard.

9. Quantification of undigested protein separated on PAGE

Protein gels were scanned with a UMAX PowerLook 1100 scanner (UMAX.com) with Photoshop 6. Gel images in TIFF format were transferred and lane profiles (intensity) or band volume (intensity × area) of prolamin bands were quantified using the Quantity One program, version 4.6.9 (Bio-Rad). Lane profiles, used to show relative amounts of each protein band separated, were expressed as intensity versus relative mobility (Rf) (**Fig. 6**). In the volume mode, volume (intensity × mm^2) was used to represent the amount of protein remaining in the gel (**Figs. 2, 4**). Higher band volume (*i.e.*, larger amount of protein) indicated lower protein digestibility and vice versa.

10. Western blots probed with antibodies against various zeins and kafirins

Equal volumes (5 µL) of pepsin-undigested extracts from *in vitro* pepsin digestion experiments were used. Protein samples were applied and separated on 12% Bis-Tris gels using MOPS buffer. Proteins were transferred to nitrocellulose membranes at 70V for 80 min at 4°C. Primary antibody (as specified in Figure legends) was used at 1:6250 dilutions in 5% powdered milk in TBS buffer incubated o/n at 4°C. Secondary antibody was goat anti-rabbit IgG-HRP conjugate (1 h at RT); membranes were developed colorimetrically with HRP substrate for 30 min. The procedure for multiple antibodies with color development was as described (Krajewski *et al.* 1996) with modifications, which included using 5% powdered milk instead of BSA and omitting the chemiluminescent detection step. All peroxidase substrates were obtained from Vector Laboratories (Burlingame, CA). The color substrates used were: anti α-zein Ab-VECTOR® SG (dark grey) or TMB (purple), anti γ-zein Ab- TMB (purple), anti β-kafirin and δ-zein Ab- DAB (brown).

RESULTS AND DISCUSSION

Separation of kafirin proteins is conventionally performed using Tris-HCl SDS-PAGE gels developed with Laemmli Tris-Glycine SDS running buffer (Hamaker *et al.* 1995). As a result, the most commonly used relative molecular mass (M_r) assignments of different kafirin types are based on this system. Bean (Bean 2003) compared separation of kafirins using precast NuPAGE Bis-Tris gels to the previously published SDS-PAGE gels (Hamaker *et al.* 1995) and concluded that precast NuPAGE Bis-Tris gels yield resolution as good as or better than traditional SDS-PAGE gradient gels. However, the NuPAGE gel system has not been widely used to analyze sorghum storage protein separation until recently (Emmambux and Taylor 2009; Wong *et al.* 2009, 2010).

Gel-based assay of *in vitro* pepsin-digested proteins using one solvent extraction

A composite of gels of *in vitro* pepsin-digested meal from six sorghum and two corn varieties differing in their digestibility is presented (**Fig. 2**). Proteins in control and undigested (0 time) residues, following extraction with borate-SDS-ME buffer, were separated on Bis-Tris gels developed with MOPS buffer, pH 7.7. Control samples not treated with pepsin (**Fig. 2**, lanes labeled C) showed a spectrum of storage proteins with M_r ranging from 15 to 200 kDa. Separation of storage proteins on these gels could generally be divided into two regions – proteins in the low M_r (15 to 30 kDa; labeled "monomers") and high M_r (40 to 200 kDa; labeled "glutelins and oligomers") range. Proteins with M_r larger than 40 kDa were glutelins and oligomers of kafirins or zeins (Nielsen *et al.* 1970; Hamaker *et al.* 1986; Wong *et al.* 2010) and represented on average about 56.9% (range 52.0 to 60.0%) of the protein volume applied to the gel. While the lower M_r proteins (15 to 30 kDa) were various monomeric forms of kafirins comprising on average about 43.0% (range 39.3 to 48.0%) of the protein volume applied to the gel (**Table 1**). Monomeric proteins were more resistant to pepsin digestion while glutelins and oligomers of kafirin were less resistant, as judged by notable differences in all sorghum varieties in the intensity of protein staining after two hours of digestion (**Table 1**). Similar differences

between the high and low M_r regions in the two corn varieties are less obvious.

Based on the M_r assignments of different kafirins in the published literature, the most abundant are α-kafirins with M_r of 22 and 25 kDa. If the α-kafirins were protected from pepsin digestion by the more resistant disulfide complexes formed by the cysteine-rich γ-kafirins (29 kDa) and β-kafirins (18 kDa) that surround them (Oria *et al.* 2000), the α-kafirins would be the major proteins left undigested. On close inspection of the gels (**Fig. 2**), more protein bands were resolved in the monomeric region than were previously reported in the literature, and the 29 kDa kafirin ('γ-kafirin') band seems to disappear (digest) faster than the α-kafirins (22 and 25 kDa). These observations raise the question about the true identity of the monomeric protein bands in **Fig. 2**.

Identification of various types of kafirins with immunoblots

A suite of monospecific zein antibodies was produced (Woo *et al.* 2001), based on genomic analysis of zeins expressed in corn endosperm. These monospecific antibodies distinguish between members of each zein family. Because zeins are very closely related to kafirins, these corn antibodies cross-react with orthologous sorghum kafirins (Wong *et al.* 2010), and therefore provide analytical tools to identify specific kafirins accurately after separation in reducing SDS-PAGE gels. This can be seen in Coomassie blue-stained gels (**Fig. 3**) of extracts of storage proteins from sorghum variety '296B' (lane A) and corn variety 'B73' (lane H). Lanes B to G (sorghum) and lanes I to N (corn) show the immunodetection of polypeptide bands by the monospecific antibodies.

The 19 kDa α-zein antibody detects multiple bands in the 'B73' corn extract, but only two bands in the sorghum extract. The two major zein bands detected had M_r of 22 and 28 kDa (**Fig. 3**, lane I); the two recognized kafirin bands were of similar M_r, 20 and 28 kDa (lane B). Reactivity of 22 kDa α-zein antibody with the corn extract was weaker than that of the 19 kDa α-zein antibody, with the former antibody only recognizing one zein band at 24.5 kDa (lane J) but two bands with the sorghum extract, 24 and 28 kDa (lane C). The 27 kDa γ-zein antibody cross-reacted with multiple bands in the 'B73' corn extract; the M_r of the major band was 27 kDa with several minor bands of higher M_rs – 43, 49, 61 and 70 kDa (lane K). While the 27 kDa γ-zein antibody recognized at least four bands in the sorghum extract, the major band was M_r 22 kDa with several minor, higher M_r bands – 43, 60, 72 and 96 kDa (lane D).

The 50 kDa γ-zein antibody recognized one protein band of 38 kDa in the 'B73' corn extract (**Fig. 3**, lane L) and at least three bands in the sorghum extract at M_r of 40, 58 and 70 kDa with decreasing reactivity (lane E). The protein bands in the sorghum extract were different from those that cross-reacted with the 27 kDa γ-zein antibody. The 10 kDa δ-zein antibody cross-reacted with two protein bands in corn at M_r of 18 and 23 kDa (lane M) and one faint band of 18 kDa in sorghum (lane F). The β-kafirin antibody recognized two very faint bands in sorghum at M_r of 15 and 17 kDa (lane G, indicated as arrows) and bands at 14 and 15 kDa with the 'B73' corn extract (lane N, indicated as arrows). There were no detectable oligomers of this kafirin under the conditions used in these experiments; however, in other experiments with different sorghum lines a dimeric form was detected (data not shown).

In **Table 2** the M_rs of the kafirin and zein bands recognized by each of the zein antibodies in **Fig. 3** are compared to published values (Woo *et al.* 2001). Our analyses reveal that the monomeric γ-kafirin migrates slightly faster and thus is smaller than the corresponding γ-zein and is smaller than most of the α-kafirins. Analysis of results also shows that α-kafirins have multiple forms with the major ones being at M_r of 23 and 29 kDa. Furthermore, α-kafirin was shown to form a dimer (see **Figs. 7B, 7E**). Demonstrating that the apparent molecular weights of major monomers of α-kafirins are larger than γ-kafirins has not been reported in the sorghum digestibility literature.

Table 2 Assignment of M_r of immunoreactive bands on western blots recognized by various monospecific and polyclonal antibodies against zeins and kafirins.

Antibody	Bis-Tris gel (Fig. 3)		Tris-glycine gel*
	Corn M_r, kDa	Sorghum M_r, kDa	B73 M_r, kDa
22 kDa α-zein	24.5#	24#, 28#, 18, 20	22#
19 kDa α-zein	22#, 28#	20#, 28#	19#, 22
27 kDa γ-zein	27#, 43, 49, 61, 70	22#, 43, 60, 72, 96	30#
50 kDa γ-zein	40, 58, 70	40, 58, 70	50
10 kDa δ-zein	18	20	10
β-kafirin	16,14	14, 12	nd

* Values of M_r cited are from Woo *et al.* (2001).
# Represents major M_r forms detected.

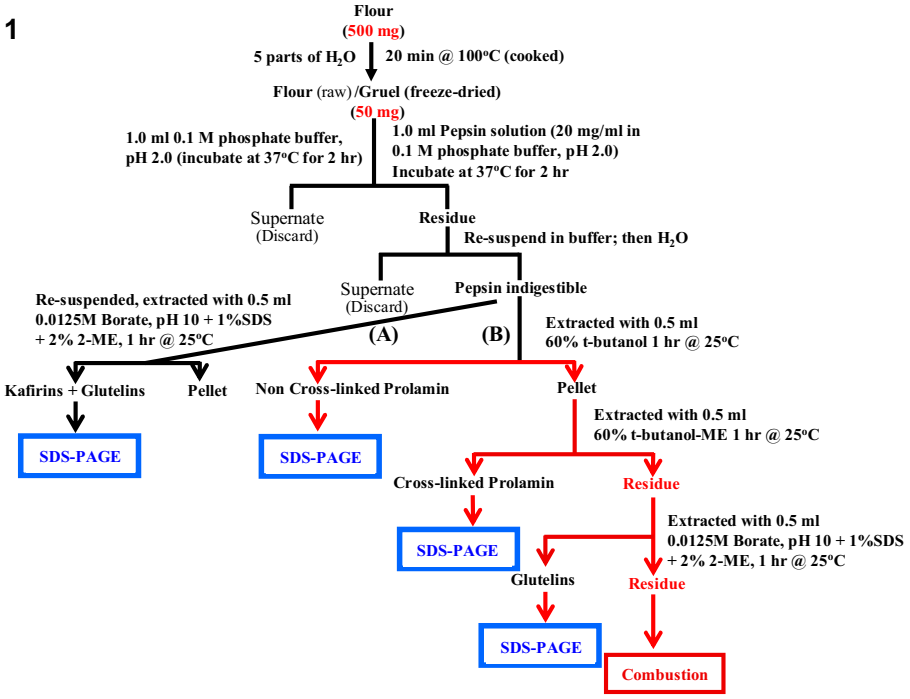
Fates of various kafirins during *in vitro* pepsin digestion

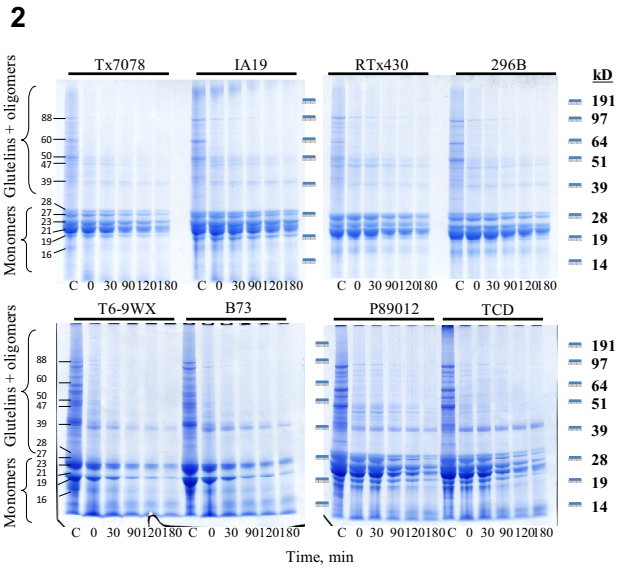
Having the ability to identify specific kafirins and to follow their fate during digestion using monospecific antibodies can lead to the development of a more in-depth understanding of the *in vitro* digestion process. A composite of gel pictures (**Fig. 4**) of four sorghum and two corn varieties, selected from **Fig. 2**, shows the fates of α-, β- and γ-kafirins (sorghum) and α-, β- and γ-zeins (corn) during a time course of *in vitro* pepsin digestion. It is well established in the literature that pepsin digestion of corn meal is faster than that of sorghum (Mertz *et al.* 1984). But for the first time we show in this study that the initial rates of disappearance of zeins are much more rapid than those of kafirins. Among the kafirins, digestion slopes appear relatively linear throughout the time course, and amounts of each kafirin are fairly consistent among the sorghum varieties with: α1 > α2 > β > γ+α. A significant finding was that γ-kafirin, which migrates with a minor form of α-kafirin, had the slowest digestion rate and that digestion of this storage protein may be a limiting factor in kafirin digestibility. This finding is still consistent with the fact that γ-kafirin is more resistant to digestion, despite the fact that the M_r of this γ-kafirin is 20 kDa rather than 29 kDa. Overall, all different types of kafirins were digested faster in the more digestible sorghum varieties ('P898012', 'TCD') than the less digestible ones ('296B', 'IA19').

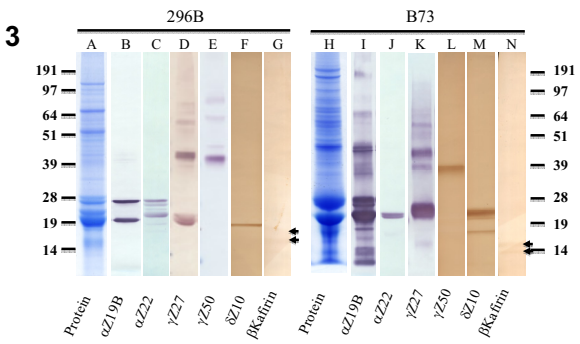
Detection of oligomers during pepsin digestion under non-reducing conditions

In addition to monomers, kafirins have been shown to exist in various oligomeric forms. These higher M_r kafirins are thought to be more resistant to pepsin digestion (Oria *et al.* 1995b; El Nour *et al.* 1998; Duodu *et al.* 2003; Nunes *et al.* 2005). One way to observe the existence of oligomers is to separate extracted proteins after pepsin digestion on SDS-PAGE gels under non-reducing conditions (Duodu *et al.* 2002; Nunes *et al.* 2004, 2005).

Four sorghum lines, 'P898012', 'TCD', '296B', and 'IA19', having differing protein digestibility characteristics, were selected from those in **Fig. 2**. Proteins were extracted from pepsin-undigested residues and then resolved on NuPAGE gels under non-reducing conditions (**Figs. 5A, 5C**). Corresponding immunoblots, using antibody against the 27 kDa γ-zein, were used to show that the most prominent oligomers were γ-kafirins that reacted with the γ-zein antibody (**Figs. 5B, 5D**). On the protein gels, it was difficult to clearly define, based solely on M_rs, which high M_r protein bands corresponded to oligomers of kafirins because some of these protein bands are glutelins (**Figs. 5A, 5C**). However, in analyzing the immunoblots made with the 27 kDa γ-zein antibody, differences among sorghum lines







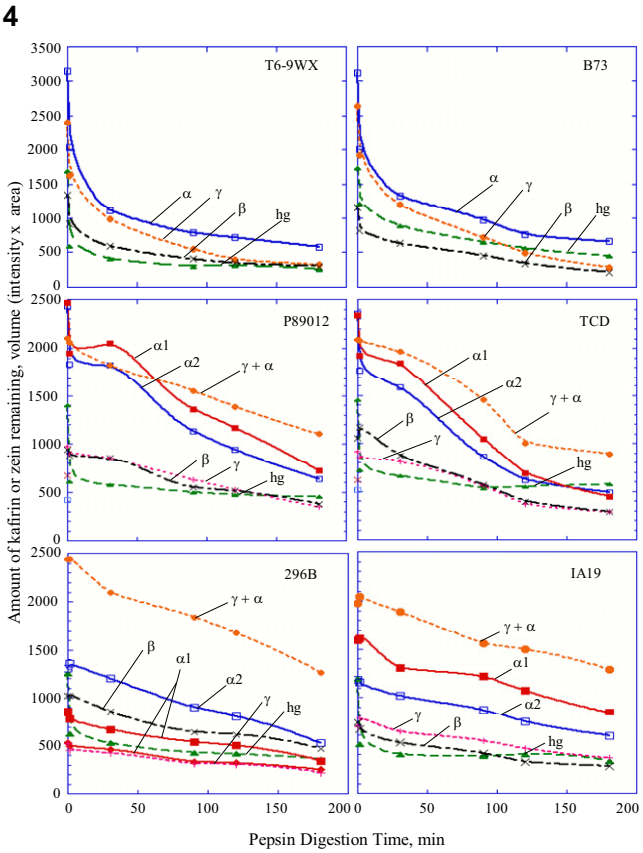


Fig. 1 Scheme of in-vitro pepsin digestion. Extraction of undigested pepsin residue when performed with 1-solvent (A) and 3-solvents (B), followed by gel-based analysis of extracted proteins.

Fig. 2 Composite of protein gels showing time course of in-vitro pepsin digestion of six sorghum and two corn lines of different protein digestibility. Undigested proteins were extracted with borate-SDS-ME buffer, pH 10 and separated on XT Bis-Tris gel that was developed with MOPS Buffer, pH 7.7. Sorghum varieties, 'Tx7078', 'IA19', 'RTx430', '296B', 'P89012', 'TCD', and corn varieties, 'T6-9WX', 'B73', indicated at the top of gel. Duration of pepsin digestion indicated at the bottom of gel. Molecular weight markers indicated in kDa on right.

Fig. 3 Composite of protein gels showing storage proteins of sorghum and corn. Stained proteins (lanes A, H) and western blots showing immuno-detection of polypeptide bands (lanes B-G; I-N) from sorghum ('296B') and corn ('B73') by monospecific zein and kafirin antibodies. Antibodies are αZ19B: 19 kDa α-zein; αZ22: 22 kDa α-zein; γZ27: 27 kDa γ-zein; γZ50: 50 kDa γ-zein; δZ10: 10 kDa δ-zein; βKafirin: β-kafirin. The color substrates used were: anti α-zein AB-VECTOR®SG (dark grey) or TMB (purple), anti γ-zein Ab- TMB (purple), anti β-kafirin and δ-zein Ab-DAB (brown). Molecular weight markers indicated in kDa on left and right.

Fig. 4 Digestion profiles of kafirins and zeins. Amount of indicated kafirins (α1, α2, β, γ, hg) remaining after various pepsin digestion times (0, 30, 90, 120, 180 min) are indicated for four sorghum ('P898012', 'TCD', '296B', 'IA19') and two corn ('T6-9WX', 'B73') lines. The band (hg) is a 39 kDa protein that resists digestion but does not cross-react with any of the antibodies tested. The identity of this protein band is not known, and might be a residual protein derived from pepsin because its presence is always detected when pepsin is added to samples.

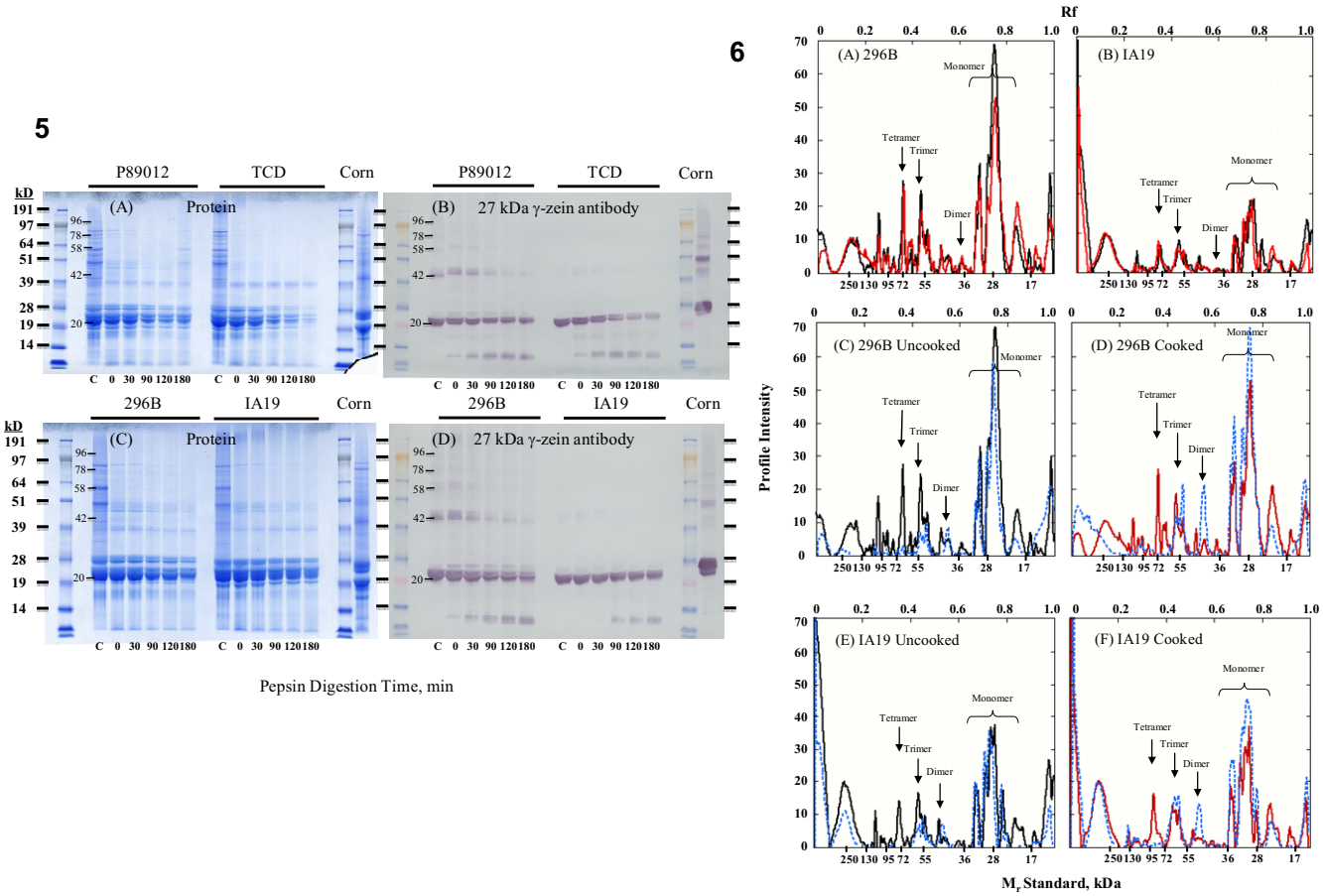


Fig. 5 Composite of protein gels showing storage proteins separated on NuPAGE gels under non-reducing conditions. Samples from four sorghum varieties ('P898012', 'TCD', '296B', 'IA19') include undigested (C) and pepsin-digested (0, 30, 90, 120, 180 min) that were separated on NuPAGE under non-reducing conditions. Gels on left (A, C) are stained. Gels on right (B, D) are westerns probed with antibody to 27 kDa γ-zein. Corn extract of 'B73' was included as reference in all four panels (A-D). Molecular weight markers indicated in kDa on left, in middle and on right. Specific molecular weights of interest are indicated as 20, 42, 58, 78, 96.

Fig. 6 Scans of NuPAGE gels showing effects of cooking on formation of oligomers. Two sorghum varieties, '296B' and 'IA19', were used. In (A) and (B), black line indicates uncooked samples, red line cooked samples. In (C) and (D), susceptibility to pepsin digestion of various oligomeric forms of kafirins in sorghum variety '296B' are profiled in uncooked (C) and cooked (D) samples. In E and F, similar profiles of sorghum variety 'IA19' are shown for uncooked (E) and cooked (F) samples. Solid line is control sample with no pepsin treatment; dashed line is 2 h pepsin-treated sample. Positions of monomers, dimmers, trimers and tetramers are indicated with bracket (monomers) and arrows. Protein intensity, indicated on y axis at left, is determined by gel scanning. Migration distance (Rf) is indicated on top. Molecular weights in kDa are indicated on x axis at bottom.

are noted. The 20 kDa monomer of γ-kafirin in 'TCD' disappeared more rapidly than in the other sorghum varieties and the dimer, trimer and tetramer forms in 'TCD' and 'IA19' were not detectable (**Figs. 5B, 5D**). The rapid disappearance of γ-kafirin and the absence of its oligomeric forms were taken as evidence that 'TCD' is the most digestible of the four sorghum lines studied. This conclusion is consistent with 'TCD''s protein digestibility (**Figs. 2, 4**). 'P898012' and '296B' appear to be intermediate in digestion (**Figs. 2, 4**) and both show evidence of dimer and trimer forms of γ-kafirin (**Figs. 5B, 5D**). Similar to 'TCD', immunoblots of 'IA19' show no detectable dimer, trimer and tetramer forms of γ-kafirin; however, unlike 'TCD', digestion of 'IA19''s 20 kDa γ-kafirin appears to be the slowest and is consistent with its slow protein digestibility (44.8%). In the case of 'IA19', it is tempting to attribute low protein digestibility to the presence of tannin in its testa layer. Tannin has been shown to inhibit enzymes and bind to γ-kafirin, rendering it harder to digest and extract (Taylor *et al.* 2007). However, 'P898012' and 'TCD' also have a testa layer with tannins. This serves as a good example to show how multiple approaches to a problem can lead to a better understanding and explanation of observations.

Effect of cooking on formation of kafirin oligomers

Cooking promotes formation and interchange of disulfide linkages among proteins with cysteine residues (Axtell *et al.* 1981; Hamaker *et al.* 1987; Oria *et al.* 1995b; Duodu *et al.* 2001, 2002) that impede digestion. Because γ-kafirins and β-kafirins are rich in cysteine residues, they are prone to form disulfide-bond polymer networks, either among like kafirins or with each other (Shull *et al.* 1992; Oria *et al.* 2000), that are protease-resistant and thus protect the more labile and centrally located α-kafirins (Oria *et al.* 1995a). In fact, it has been stated that the amount of high M_r oligomers formed is proportional to the degree of indigestibility (Duodu *et al.* 2002; Nunes *et al.* 2005; Byaruhanga *et al.* 2006).

The effect of cooking on the formation of high M_r oligomers of kafirin in '296B' and 'IA19' is shown in **Figs. 6A** and **6B**, respectively. The effects of pepsin digestion on the fate of monomers and oligomers of kafirins were also studied in uncooked and cooked samples of '296B' (**Figs. 6C** vs. **6D**) and 'IA19' (**Figs. 6E** vs. **6F**), respectively. Cooking is shown to promote formation of disulfide-linked oligomers and polymers; preliminary evidence suggests that α- and β-kafirins can form dimers but other oligomers are predominantly formed by γ-kafirins. But the proportion of oligomers formed is relatively minor compared with the major monomeric regions (**Figs. 6A, 6B**), so it is premature to conclude that the formation of indigestible oligomers is related to protein indigestibility and further investigation is needed. The methods and procedures described in this paper will provide the tools necessary to better understand the impact of oligomer/polymer formation on the digestibility

of sorghum.

The one solvent extraction protocol described above yielded interesting information about the variability in digestion of various kafirins from different sorghum varieties. The γ-kafirins represent a small percentage of total kafirins yet seem to be present in the pepsin-undigested residues under several circumstances, while the α-kafirins, a multi-gene family that make up 80% of the total kafirins, do not. This suggests the idea that α-kafirins might participate in forming oligomeric networks with themselves and/or with other kafirins.

Sequential extraction by three solvents of pepsin-undigested residues of uncooked and cooked sorghum and corn meal

El Nour *et al.* (1998) showed that kafirins exist in oligomeric forms, when meal is extracted using 60% *t*-butanol as the extraction solvent. Based on their work and others (El Nour *et al.* 1998; Nunes *et al.* 2004, 2005), we adopted a sequential, three-solvent system to extract the protein remaining undigested by pepsin. We used this approach (**Fig. 1**, path B) to attempt to assess the relative contributions of the various oligomeric forms of kafirin toward sorghum's indigestibility. In addition this approach provides an opportunity to determine how cooking affects formation of oligomers and their subsequent susceptibility to pepsin digestion. The three-solvent system was used to extract proteins from uncooked (**Fig. 7**) and cooked (**Fig. 8**) sorghum and corn samples, both before and after 2 h pepsin digestion (**Fig. 1**, path B). Results are presented in the form of protein gels and immunoblots.

1. Extraction with 60% t-butanol

Extraction with 60% *t*-butanol was used to extract prolamins in their native state to determine if any oligomeric forms of prolamin existed in uncooked samples. Several groups of proteins were observed in the sorghum residues, yielding approximately similar amounts of protein in low M_r (14 to 28 kDa) and mid M_r (45 to 51 kDa) regions, with a trace amount in the higher M_r regions, *i.e.*, 60 to 70 kDa, ≥ 95 kDa (**Fig. 7A**); extracts of corn showed exceptions (lanes 5 and 6, see below). Sorghum lines 'P898012' and 'TCD' had more proteins extracted in the low and mid M_r regions relative to sorghum varieties '296B' and 'IA19' (**Fig. 7A**, lanes 1 and 2 vs. 3 and 4). The extracted oligomeric forms (45 to 51 kDa) seemed to be more resistant to pepsin digestion than the monomeric forms (**Fig. 7A**, lanes 7-10 +pepsin vs. 1-4 -pepsin). Corn was very different from sorghum. Much more protein was extracted with 60% *t*-butanol from the undigested meal and also higher quantities and more forms of zein oligomers were observed than in sorghum (**Fig. 7A**, lanes 5 and 6 vs. lanes 1 to 4). Zein oligomers disappeared almost completely following 2 h pepsin treatment (**Fig. 7A**, lanes 11 and 12).

Notably, cooking affects the extractability of the oligomers. Trace amounts of kafirin oligomers were extracted with 60% *t*-butanol from cooked samples of sorghum (**Fig. 8A**, lanes 1-4), and much lesser amounts of proteins were extracted from cooked samples of corn (**Fig. 8A**, lanes 5 and 6), under the same conditions. The zein oligomers were much more susceptible to pepsin digestion (**Fig. 8A**, lanes 11 and 12) relative to those of sorghum samples (**Fig. 8A**, lanes 7 to 10). These observations corroborate the fact that cooking affects sorghum protein digestibility much more than that of corn (Mertz *et al.* 1984).

2. Immunoreactivity of proteins extracted with 60% t-butanol

Polypeptides extracted with 60% *t*-butanol showed visible immunoreactivity with the 22 kDa α-zein antibody but not with 27 kDa γ-zein antibody in both uncooked (**Figs. 7B** vs. **7C**) and cooked (**Fig. 8B** vs. **8C**) samples. This indicates that the polypeptides extracted with 60% *t*-butanol in sorghum and corn were made up predominantly of α-kafirin and α-zein subunits, respectively.

3. Extraction with 60% t-butanol plus 2-ME

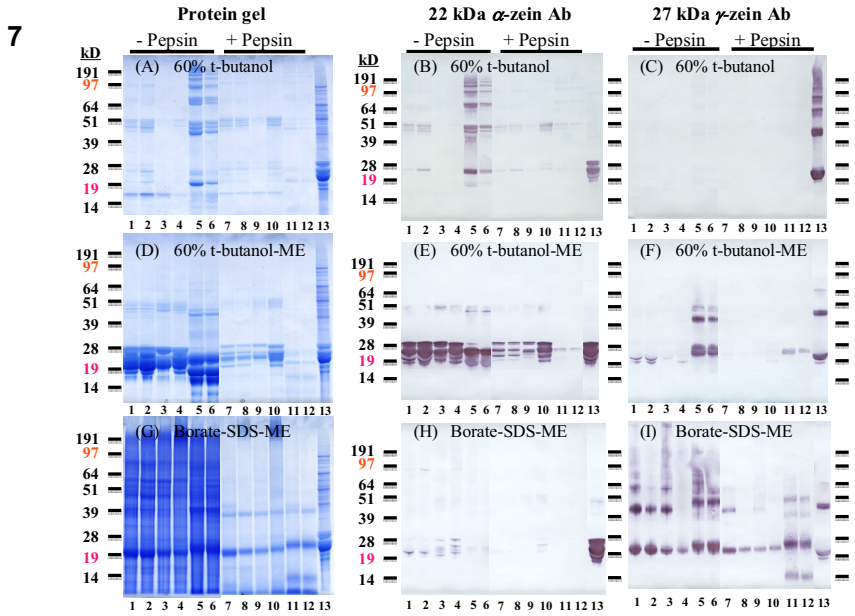
The addition of 2-ME to this extraction procedure is used to reduce disulfide linkages between prolamin subunits and improve their solubility in 60% *t*-butanol. Thus, a majority of kafirins was extracted (**Figs. 7D, 8D**), indicating that the cross-linking affected their solubility in *t*-butanol. Two groups of proteins were extracted with this procedure. The major group, with M_r ranging from 16 to 28 kDa, represents the monomeric forms of kafirin or zein and this group is much more extractable with this solvent than with *t*-butanol alone (**Figs. 7D, 8D** vs. **Figs. 7A, 8A**). A minor group, with M_r around 45 to 51 kDa, include the dimeric forms of kafirins and zeins (**Fig. 7D**, lanes 1 to 6), which appear to resemble proteins of similar M_r extracted with 60% *t*-butanol alone (**Fig. 7A**, lanes 1 to 6). Kafirin proteins with M_r above 60 kDa were not extractable under these conditions. The extracted dimeric kafirins showed differential susceptibility to pepsin digestion. In three out of four uncooked sorghum samples analyzed (**Fig. 7D**, lanes 1 to 4 vs. lanes 7 to 10), the monomeric forms were very susceptible to pepsin digestion except 'IA19' (lane 10). All forms of zein in uncooked corn were extremely digestible (**Fig. 7D**, lanes 5 and 6 vs. 11 and 12). Dimeric forms of kafirin also digested readily to a certain level (**Fig. 7D**, lanes 1 to 4 vs. 7 to 10), to a lesser extent with 'IA19' (lane 10).

As judged by staining intensity, similar amounts of the monomeric, as well as dimeric, forms of kafirins, are recovered from cooked vs. uncooked samples, when extracted with the addition of 2-ME (**Fig. 8D**, lanes 1 to 4 vs. **Fig. 7D**, lanes 1 to 4) but they are more resistant to pepsin digestion when cooked (**Fig. 8D**, lanes 7 to 10 vs. **Fig. 7D**, lanes 7 to 10). This observation suggests that heating affects kafirin structure causing the proteins to become more resistant to pepsin digestion in the four sorghum lines analyzed. This observation is in contrast to corn where any structural change caused by heating does not seem to affect zein protein digestibility (**Figs. 8D** vs. **7D**, lanes 5 and 6 vs. lanes 11 and 12).

4. Immunoreactivity of proteins extracted with 60% t-butanol-ME

Most monomeric forms of kafirin (18 to 28 kDa) in the undigested, uncooked meal cross-reacted strongly with the 22 kDa α-zein antibody with the characteristic M_rs at 28 and 23 kDa and with fainter reactivity at 20 and 19 kDa (**Fig. 7E**, lanes 1 to 4). The faint reactivity of the kafirins with the 27 kDa γ-zein antibody (**Fig. 7F**, lanes 1 to 4) strongly suggests that most proteins extracted with 60% *t*-butanol-ME in uncooked samples were α-kafirins that existed in cross-linked forms because they were not extracted in *t*-butanol alone. Cooking may promote some interaction between α- and γ-kafirin as indicated in **Fig. 8F**, lanes 1 to 3. The trace reactivity of dimeric forms of kafirin at 50 kDa with the 22 kDa α-zein antibody, but not with the 27 kDa γ-zein antibody (**Fig. 7E**, lanes 1 to 4 vs. **Fig. 7F**, lanes 1 to 4), suggests that α-kafirin can form dimers. In summary, proteins extracted with 60% *t*-butanol-ME were comprised of predominantly α-kafirins.

Different observations were found in corn. In addition to cross-reaction with the 22 kDa α-zein antibody, a significant amount of extracted protein also showed cross-reactivity with the 27 kDa γ-zein antibody in both the monomeric and dimeric regions in uncooked (**Figs. 7E** vs. **7F**, lanes 5 and 6) and cooked samples (**Figs. 8E** vs. **8F**, lanes 5 and 6), although the proteins were very susceptible to pepsin digestion in both uncooked (**Figs. 7E** and **7F**, lanes 11 and 12) and cooked samples (**Figs. 8E** vs. **8F**, lanes 11 and 12). It is important to note that 60% *t*-butanol-ME did not appear to extract γ-kafirin effectively.



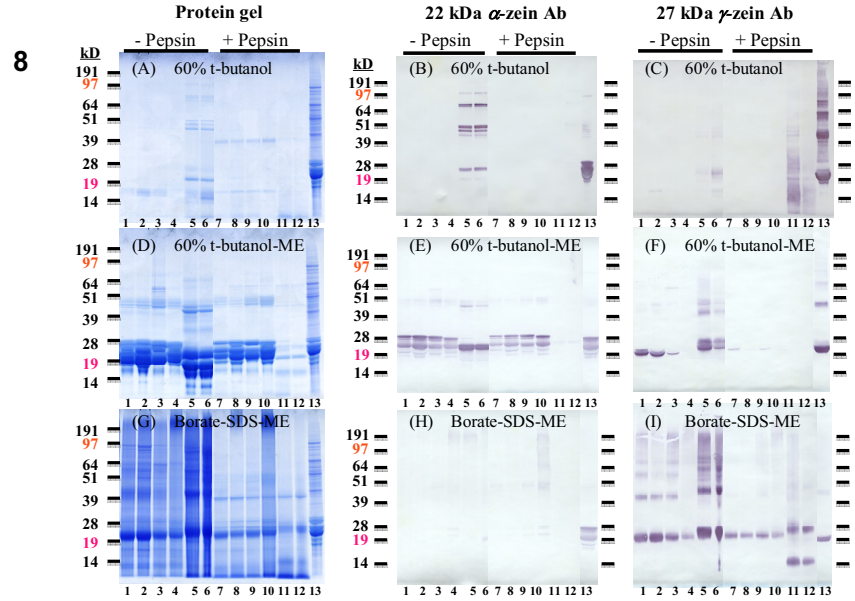


Fig. 7 Composite of protein gels and immunoblots of residual proteins following pepsin digestion of uncooked sorghum and corn samples. Samples with (+) and without (-) pepsin were extracted sequentially with 60% *t*-butanol (A to C), 60% *t*-butanol + 2-ME (D to F), and borate-SDS-ME (G to I). Extracts were precipitated and pellets redissolved; equal volumes were separated on Criterion XT gel under non-reducing conditions. Samples are 'P898012': lanes 1, 7; 'TCD': lanes 2, 8; '296B': lanes 3, 9; 'IA19': lanes 4, 10; 'B73' (corn): lanes 5, 11; 'T6-9WX' (corn): lanes 6, 12; reference is 'P898012', borate-SDS-ME extract dissolved in reducing buffer: lane 13. Stained protein gels are presented in panels A, D, G; westerns using 22 kDa α-zein antibody in panels B, E, H; westerns using 27 kDa γ-zein antibody in panels C, F, I. The major protein band at 39 kDa is the same as hg in Fig. 4. Molecular weights in kDa are indicated on left of each gel and far right.

Fig. 8 Composite of protein gels and immunoblots of residual proteins following pepsin digestion of cooked sorghum and corn samples. Samples with (+) and without (-) pepsin were extracted sequentially with 60% *t*-butanol (A to C), 60% *t*-butanol + 2-ME (D to F), and borate-SDS-ME (G to I). Extracts were precipitated and pellets redissolved; equal volumes were separated on Criterion XT gels under non-reducing conditions. Samples are numbered as in **Fig. 7**. Stained protein gels are presented in panels A, D, G; westerns using 22 kDa α-zein antibody is panels B, E, H; westerns using 27 kDa γ-zein antibody in panels C, F, I. Molecular weights in kDa are indicated on left of each gel and on far right.

5. Borate-SDS-ME extraction

This solvent is traditionally used to extract cross-linked glutelins (M_r >40 kDa) after dilute salt and alcohol extractions (Hamaker *et al.* 1986) and is the same solvent used for the one-solvent extraction (**Fig. 2**). Glutelins contain large numbers of disulfide-linked polypeptides relative to albumins, globulins and kafirins (Nielsen *et al.* 1970). Using this borate solvent extraction method, large numbers of proteins were extracted with several prominent bands in the M_rs range of 39 to 97 kDa. Considerable smearing occurred when the gel was run under non-reducing condition, when samples were not denatured by boiling in the presence of 2-ME. The 'glutelin' fraction in both sorghum and corn showed no differential susceptibility to pepsin digestion in uncooked samples, as all protein bands were easily digested with the exception of two protein bands, *i.e.*, 39 and 20 kDa (**Fig. 7G**, lanes 1 to 6 vs. 7 to 12). Cooked samples had fewer extracted proteins (less intense staining); this might be due to aggregation promoted by high temperatures that rendered proteins less extractable (**Fig. 8G**, lanes 1to 6 vs. **Fig. 7G**, lanes 1 to 6). But, digestion by pepsin of the cooked samples appears almost equal to that of uncooked samples (**Fig. 8G**, lanes 7 to 12 vs. **Fig. 7G**, lanes 7 to 12).

Of the two resistant protein bands, the major one, around 20 and 27 kDa for sorghum and corn respectively, was present in all control samples and was resistant to pepsin digestion in all uncooked sorghum and corn samples

Table 3 Percent protein N in residues after 3-solvent sequential extraction.

Sample	Uncooked		Cooked	
	% Protein N		% Protein N	
	Control	Post-pepsin	Control	Post-pepsin
'P89012'	2.69	2.41	2.50	2.25
'TCD'	2.19	2.22	2.72	2.44
'296B'	3.88	3.13	5.03	3.88
'IA19'	4.19	4.03	5.84	5.19
'B73'	2.31	2.25	2.25	1.84
'T6-9WX'	2.53	2.03	3.44	3.06

(**Fig. 7G**, lanes 1 to 6 vs. lanes 7 to 12). The other band, M_r around 36 to 38 kDa, which does not appear in control samples (lane 13), was less abundant than the 20 kDa protein in both uncooked (**Fig. 7A**, lanes 7 to 12) and cooked (**Fig. 8G**, lanes 7 to 12) samples of sorghum and corn. The identity of this protein band is not known, and might be a residual protein related to pepsin because its presence is always observed where pepsin is added.

6. Immunoreactivity of proteins extracted with borate-SDS-ME

The 27 kDa γ-zein antibody cross-reacted strongly with several extracted protein bands at 20, 43 and 58 kDa in all uncooked sorghum and corn lines except 'IA19' (**Fig. 7I**, lanes 1 to 3, 5 and 6 vs. 4). These bands represent the monomeric, dimeric and trimeric forms of γ-kafirins that were readily extracted with this solvent system. This observation is important in that it explains why γ-kafirin is absent when extracting with 60% *t*-butanol and 60% *t*-butanol with 2-ME; because γ-kafirin and its oligomers are readily extracted in this solvent, as reported previously (Evans *et al.* 1987). There was a minor presence of α-kafirins in this extracted fraction of uncooked samples, as evidenced by the slight reactivity with the 22 kDa α-kafirin antibody (**Fig. 7H**, lanes 1 to 4); similar results were found in the cooked samples (**Figs. 8I** vs. **8H**, lanes 1 to 4). Immunoblot analyses reveal that native dimeric forms of α-kafirins exist but the majority of dimer, trimer and tetramer forms that exist are made up predominantly of γ-kafirins (**Figs. 7I** and **8I**, lanes 1 to 6).

Even after using the three-solvent extraction system, extraction is not complete. **Table 3** shows 2 to 5 % of protein remains in the residues after sequential extractions with the three solvents. And it is notable that the more indigestible 'IA19' variety has more of these residues than the other more digestible varieties. Identification of these un-extractable proteins requires further investigation.

CONCLUDING REMARKS

The extent of kafirin digestion has long been associated with total digestibility of sorghum meal. The gel-based, one-solvent extraction of pepsin-undigested residues used previously gives an overall approximation of digestion rates of sorghum meal. The superior resolution of individual proteins with the Bis-Tris gel system showed that α-, β-, γ- and δ-kafirins are digested at different rates in the pepsin indigestible residues. Immunoblot analysis revealed that the most predominant protein band is a γ-kafirin with an M_r lower than most α-kafirins. The α-kafirin family is observed as multiple bands reflecting that they are products of a multigene family. They can be detected in dimer form; however, γ-kafirins represent most of the oligomeric forms identified when using a one-solvent extraction system.

Gel-based analysis of extracted proteins using the three-solvent system adds more detailed information than the one-solvent system. Through the use of the three-solvent system, we have shown that α-kafirins are more hydrophobic than γ-kafirins because most are extracted in 60% *t*-butanol in the presence of 2-ME. A small percentage of the α-kafirins exist as dimers extractable without a reductant, but the majority exists as disulfide-linked forms, causing their extraction to require the addition of a reducing agent during butanol extraction. γ-Kafirins are, however, preferentially extracted in Borate-SDS-ME buffer, pH10. Because of the differences in their solvent solubility, the use of the Borate-SDS-ME buffer (one-solvent extraction) favors the extraction of γ-kafirins over α-kafirins, a feature that likely explains why both the monomeric and oligomeric forms of γ-kafirins are more protease resistant than α-kafirin, i.e., the buffer is preferentially extracting more γ-kafirins than α-kafirins. This observation argues for the need to use the 3-solvent system to crosscheck findings related to specific kafirin digestibility.

Cooking promotes the formation of disulfide linked oligomers and polymers, especially among γ-, β- and α-kafirins. But the proportion of these oligomers formed is relatively minor when compared with the monomeric forms. Further investigation is, therefore, needed to establish whether the formation of indigestible oligomers is proportional to negative effects on protein digestibility. The analytical systems described in this study provide a useful set of tools to dissect the role of these kafirin oligomers and polymers in digestibility.

Sorghum, like all crops, is subject to environmental influences that can affect the digestibility of the grain. In order to make improvements in grain protein digestibility, a thorough understanding is needed of how various types of storage proteins form and interact and how they behave when subjected to digestion. The approaches used in this study provide a systematic set of tools to analyze in detail how the formation and interaction of specific kafirins affect their behavior during pepsin digestion. The knowledge gained will lead to a better understanding of the mechanisms involved in protein digestibility, thereby making possible the manipulation of identified genes responsible for protein formation and composition to delineate further their effects on digestibility. Thus, new conventional or molecular strategies to improve sorghum digestibility may be devised.

ACKNOWLEDGEMENTS

At an early stage, these studies were supported by the U.S. Agency for International Development and the Bill and Melinda Gates Foundation. PGL was supported by the USDA Cooperative Extension Service, and BBB by the Agricultural Experiment Station through UC Berkeley. We thank Dr. R. Jung, Pioneer Hi-Bred for mono-specific zein antibodies; Dr. B.A. Larkins for antibody against α-zein; and Dr. B. R. Hamaker for antibody against β-kafirin. Numerous undergraduate students also made contributions, especially Joseph Ahn, Youngin Lee, Won Kyung Lee, Hyun Ji Kang, and Stephanie Wu.

DISCLAIMER

REFERENCES

Aboubacar A, Axtell JD, Huang CP, Hamaker BR (2001) A rapid protein digestibility assay for identifying highly digestible sorghum lines. *Cereal Chemistry* **78**, 160-165

Axtell JD, Kirleis A, Hassen MM, D'Croz-Mason N, Mertz ET, Munck L (1981) Digestibility of sorghum proteins. *Proceedings of the National Academy of Sciences USA* **78**, 1333-1335

Bean SR (2003) Evaluation of novel pre-cast gradient gels for the separation of sorghum proteins. *Cereal Chemistry* **80**, 500-504

Belton PS, Delgadillo I, Halford NG, Shewry PR (2006) Kafirin structure and functionality. *Journal of Cereal Science* **44**, 272-286

Byaruhanga YB, Emmambux MN, Belton PS, Wellner N, Ng KG, Taylor JRN (2006) Alteration of kafirin and kafirin film structure by heating with microwave energy and tannin complexation. *Journal of Agricultural and Food Chemistry* **54**, 4198-4207

Chandrashekar A, Kirleis AW (1988) Influence of protein on starch gelatinization in sorghum. *Cereal Chemistry* **65**, 457-462

Coleman CE, Yoho PR, Escobar S, Ogawa M (2004) The accumulation of α-zein in transgenic tobacco endosperm is stablilized by co-expression of β-zein. *Plant and Cell Physiology* **45**, 864-871

Duodu KG, Nunes A, Delgadillo I, Parker ML, Mills ENC, Belton PS, Taylor JRN (2002) Effect of grain structure and cooking on sorghum and maize *in vitro* protein digestibility. *Journal of Cereal Science* **35**, 161-174

Duodu KG, Tang H, Grant A, Wellner N, Belton PS, Taylor JRN (2001) FTIR and solid state ^{13}C spectroscopy of proteins of wet cooked and popped sorghum and maize. *Journal of Cereal Science* **33**, 261-269

Duodu KG, Taylor JRN, Belton PS, Hamaker BR (2003) Factors affecting sorghum protein digestibility. *Journal of Cereal Science* **38**, 117-131

El Nour INA, Peruffo ADB, Curioni A (1998) Characterisation of sorghum kafirins in relation to their cross-linking behaviour. *Journal of Cereal Science* **28**, 197-207

Elkhalifa AEO, Chandrashekar A, Mohamed BE, El Tinay AH (1999) Effect of reducing agents on the *in vitro* protein and starch digestibilities of cooked sorghum. *Food Chemistry* **66**, 323-326

Emmambux MN, Taylor JRN (2009) Properties of heat-treated sorghum and maize meal and their prolamin proteins. *Journal of Agricultural and Food Chemistry* **57**, 1045-1050

Evans DJ, Schussler L, Taylor JRN (1987) Isolation of reduced-soluble protein from sorghum starchy endosperm. *Journal of Cereal Science* **5**, 61-65

Ezeogu LI, Duodu KG, Taylor JRN (2005) Effects of endosperm texture and cooking conditions on the *in vitro* starch digestibility of sorghum and maize flours. *Journal of Cereal Science* **42**, 33-44

Garratt R, Oliva G, Caracelli I, Leite A, Arruda P (1993) Studies of the zein-like α-prolamins based on an analysis of amino acid sequences: Implications for their evolution and three-dimensional structure. *Proteins* **15**, 88-99

Hamaker BR, Bugusu BA (2003) Sorghum proteins and food quality. In: Belton PS, Taylor JRN (Eds) *Workshop on the Proteins of Sorghum and Millets: Enhancing Nutritional and Functional Properties for Africa* [CD]. AFRIPRO, Pretoria, South Africa

Hamaker BR, Kirleis AW, Butler LG, Axtell JD, Mertz ET (1987) Improving the *in vitro* protein digestibility of sorghum with reducing agents. *Proceedings of the National Academy of Sciences USA* **84**, 626-628

Hamaker BR, Kirleis AW, Mertz ET, Axtell JD (1986) Effect of cooking on the protein profiles and *in vitro* digestibility of sorghum and maize. *Journal of Agricultural and Food Chemistry* **34**, 647-649

Hamaker BR, Mohamed AA, Habben JE, Huang C-P, Larkins BA (1995) An efficient procedure for extracting maize and sorghum kernel proteins reveals higher prolamin contents than the conventional method. *Cereal Chemistry* **72**, 583-588

Johns CO, Brewster JF (1916) Kafirin, an alcohol-soluble protein from kafir, *Andropogon sorghum*. *The Journal of Biological Chemistry* **28**, 59-65

Kim CS, Woo Y-M, Clore AM, Burnett RJ, Carneiro NP, Larkins BA (2002) Zein protein interactions, rather than the asymmetric distribution of zein mRNAs on endoplasmic reticulum membrane, influence protein body formation in maize endosperm. *Plant Cell* **14**, 655-672

Krajewski S, Zapata JM, Reed JC (1996) Detection of multiple antigens on western blots. *Analytical Biochemistry* **236**, 221-228

Laidlaw HKC, Mace ES, Williams SB, Sakrewski K, Mudge AM, Prentis PJ, Jordan DR, Godwin ID (2010) Allelic variation of the β-, γ- and δ-kafirin genes in diverse sorghum genotypes. *Theoretical and Applied Genetics* **121**, 1227-1237

Lending CR, Larkins BA (1989) Changes in the zein composition of protein bodies during maize endosperm development. *Plant Cell* **1**, 1011-1023

Mazhar H, Chandrashekar A, Shetty HS (1993) Isolation and immunochemical characterization of alcohol-extractable proteins (kafirins) of *Sorghum bicolor* (L.) Moench. *Journal of Cereal Science* **17**, 83-93

Mertz ET, Hassen MM, Cairn-Whittern C, Kirleis AW, Tu L, Axtell JD (1984) Pepsin digestibility of proteins in sorghum and other major cereals. *Proceedings of the National Academy of Sciences USA* **81**, 1-2

Micro-Dumas combustion method (1997) Elemental analysis by micro-Dumas combustion. Available online: http://www.uga.edu/sisbl/udumas.html

Mosse J (1990) Nitrogen to protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed protein content. *Journal of Agricultural and Food Chemistry* **38**, 18-24

Nielsen HC, Paulis JW, James C, Wall JS (1970) Extraction and structure studies on corn glutenin proteins. *Cereal Chemistry* **47**, 501-512

Nunes A, Correia I, Barros A, Delgadillo I (2004) Sequential *in vitro* pepsin digestion of uncooked and cooked sorghum and maize samples. *Journal of Agricultural and Food Chemistry* **52**, 2052-2058

Nunes A, Correia I, Barros A, Delgadillo I (2005) Characterization of kafirin and zein oligomers by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Agricultural and Food Chemistry* **53**, 639-643

Oria MP, Hamaker BR, Axtell JD, Huang C-P (2000) A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm protein bodies. *Proceedings of the National Academy of Sciences USA* **97**, 5065-5070

Oria MP, Hamaker BR, Shull JM (1995a) *In vitro* protein digestibility of developing and mature sorghum grain in relation to α-, β- and γ-kafirin disulfide crosslinking. *Journal of Cereal Science* **22**, 85-93

Oria MP, Hamaker BR, Shull JM (1995b) Resistance of sorghum α-, β- and γ-kafirins to pepsin digestion. *Journal of Agricultural and Food Chemistry* **43**, 2148-2152

Shewry PR (2007) Improving the protein content and composition of cereal grain. *Journal of Cereal Science* **46**, 239-250

Shull JM, Chandrashekar A, Kirleis AW, Ejeta G (1990) Development of sorghum [*Sorghum bicolor* (L.) Moench] endosperm in varieties of varying hardness. *Food Structure* **9,** 253-267

Shull JM, Watterson JJ, Kirleis AW (1991) Proposed nomenclature for the alcohol-soluble proteins (kafirins) of *Sorghum bicolor* (L. Moench) based on molecular weight, solubility, and structure. *Journal of Agricultural and Food Chemistry* **39**, 83-87

Shull JM, Watterson JJ, Kirleis AW (1992) Purification and immunocytochemical localization of kafirins in *Sorghum bicolor* (L. Moench) endosperm. *Protoplasma* **171**, 64-74

Taylor J, Bean SR, Ioerger BP, Taylor JRN (2007) Preferential binding of sorghum tannins with γ-kafirin and the influence of tannin binding on kafirin digestibility and biodegradation. *Journal of Cereal Science* **46**, 22-31

Taylor JRN, Novellie L, Liebenberg NVDW (1984) Sorghum protein body composition and ultrastructure. *Cereal Chemistry* **61**, 60-73

Taylor JRN, Schussler L, Liebenberg NVDW (1985) Protein body formation in the starchy endosperm of developing *Sorghum bicolor* (L.) Moench seeds. *Suid Afrikaanse Tydskrift vir Plantkunde* **51**, 81-86

Watterson JJ, Shull JM, Kirleis AW (1993) Quantitation of α-, β- and γ-kafirins in vitreous and opaque endosperm of *Sorghum bicolor*. *Cereal Chemistry* **70**, 452-457

Wong J, Lau T, Cai N, Singh J, Pedersen JF, Wilson J, Vensel WH, Hurkman WJ, Lemaux PG, Buchanan BB (2009) Digestibility of protein and starch from sorghum (*Sorghum bicolor*) is linked to biochemical and structural features of grain endosperm. *Journal of Cereal Science* **49**, 73-82

Wong JH, Marx DB, Wilson JD, Buchanan BB, Lemaux PG, Pedersen JF (2010) Principal component analysis and biochemical characterization of protein and starch reveal primary targets for improving sorghum grain. *Plant Science* **179**, 598-611

Woo Y-M, Hu DW-N, Larkins BA, Jung HR (2001) Genomics analysis of genes expressed in maize endosperm identifies novel seed proteins and clarifies patterns of zein gene expression. *Plant Cell* **13**, 2297-2317