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Principal component analysis and biochemical characterization of protein and starch reveal primary targets for improving sorghum grain[☆]

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ABSTRACT

Limited progress has been made on genetic improvement of the digestibility of sorghum grain because of variability among different varieties. In this study, we applied multiple techniques to assess digestibility of grain from 18 sorghum lines to identify major components responsible for variability. We also identified storage proteins and enzymes as potential targets for genetic modification to improve digestibility. Results from principal component analysis revealed that content of amylose and total starch, together with protein digestibility (PD), accounted for 94% of variation in digestibility. Control of amylose content is understood and manageable. Up-regulation of genes associated with starch accumulation is clearly a future target for improving digestibility. To identify proteins that might be targets for future modification, meal from selected lines was digested *in vitro* with pancreatin in parallel with pepsin and α-amylase. The %PD was influenced by both the nature of the protein matrix and protein body packaging. Owing to its ability to form oligomers, the 20 kDa γ-kafirin was more resistant to digestion than counterparts lacking this ability, making it a target for down-regulation. Greater understanding of interactions among the three traits identified by principal component analysis is needed for both waxy and non-waxy varieties.

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1. Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is a major crop worldwide with many notable attributes. A C4 plant, sorghum grows well in tropical climates, is efficient in water usage and tolerates drought as well as waterlogged and saliferous soils. This capability makes sorghum better able to grow on marginal lands that do not support maize (*Zea mays* L.) and other cereal crops [1]. Its utility as human food and animal feed is, however, not maximal because of

decreased protein and starch digestibility, especially after cooking [2–4].

The most abundant component (ca. 70%) of the dry grain is starch [5–7], which is composed of two polymers of glucose, amylose and amylopectin. Amylose is a linear polymer of glucose, linked through α-1,4-glycosidic bonds. Amylopectin is a branched polymer of glucose, composed of linear α-1,4-glycosidic linkages as with amylose, but with a branch point made of α-1,6-glycosidic linkages. Starch is packaged as semicrystalline granules within the endosperm, with the shape, size and content of amylose and amylopectin varying within the cultivars. In sorghum grain, starch is typically composed of a mixture of 30% amylose and 70% amylopectin [5]. Grain that yields starch of this type is referred to as non-waxy. However, there are grains that lack or have inactive granule bound starch synthase, GBSS1 [8] – the enzyme that synthesizes amylose [9] – and thus produce low amounts of this polymer. Such low amylose grains exhibit endosperm fracture patterns resembling those of wax and are thus referred to as "waxy." Starch of waxy and non-waxy grain has different gelatinization and digestibility properties and, therefore, different product utilities [10,11].

The sorghum grain or kernel contains three parts: the protective outer pericarp, embryo (germ) and endosperm. The distribution of starch granules within these parts varies with grain type and culti-

var. In general, the pericarp and embryo together represent a small percentage of the kernel and contain little starch. Most of the starch resides in the endosperm – a compartment that can be divided into an outermost aleurone layer, the peripheral endosperm (subaleurone layer), the underlying corneous endosperm and the innermost, floury endosperm. The aleurone cells lack starch granules but contain autolytic enzymes, like amylases and protease inhibitors, as well as water-soluble vitamins and minerals and spherical bodies containing protein and lipid. Starch granules in the peripheral and corneous endosperm are surrounded by numerous protein bodies and are embedded in a dense matrix of dried endosperm cells. The matrix, consisting mainly of protein and non-starch carbohydrates, is relatively impervious to water and hydrolytic enzymes. In contrast, the floury endosperm has little cell structure and the highest density of starch granules, which are more accessible to enzymatic hydrolysis than those in the peripheral and corneous endosperm [12,6].

Relative proportions of peripheral, corneous and floury endosperm vary among sorghum varieties. Grain cultivars, with kernels having a high proportion of peripheral and corneous endosperm, are termed vitreous, corneous or flinty because of their glassy appearance; they are harder to digest. Kernels containing high proportions of floury endosperm are chalky in appearance and are termed floury, opaque or soft; they are easier to digest [5,13,14].

Protein on average makes up 12% of grain dry weight. Most literature reports indicate that digestibility of sorghum grain is controlled by protein body structure and location of the seed storage proteins, kafirins [15,16]. Protein bodies house kafirins, which comprise 70–80% of the protein in whole grain sorghum flour [15]. The principal storage protein, α-kafirin, comprising ∼80% of total kafirins, is centrally located in the protein body and is encapsulated by β- and γ-kafirins found on the periphery of the spherical protein body. Cysteine-rich β- and γ-kafirins tend to form disulfide-bonded, protease-resistant complexes that retard digestion of the more digestible α-kafirins [16,17]. Earlier research showed that kafirins are the last proteins to be digested, in particular, α-kafirins [4,16,18]. Cooking decreases the digestibility of sorghum flour and enhances the formation of extensive disulfide crosslinking due mainly to γ-kafirins [4]. Addition of reducing agents breaks these bonds and enhances digestibility of both uncooked and cooked flour [19–21]. The denser arrangement of protein bodies and protein matrix around starch granules in the corneous endosperm, relative to the floury endosperm, has been cited as a barrier to amylase attack that causes a reduction in starch digestibility and gelatinization [22–24,14,25–27].

Numerous methods have been employed to assess protein and starch digestibility in sorghum. Each method was developed for a particular use and has strengths and weaknesses. In vitro dry matter digestion (IVDMD) uses bovine (Bos tarus L.) ruminal fluid as the source of digestive enzymes and thus mimics the digestive environment of the rumen – a second stomach of ruminants in which contents are almost continuously mixed with those of the first stomach, or reticulum. Both stomachs share a dense population of microorganisms, i.e., bacteria, protozoa, and fungi [6,28]. The rumen is considered to be a large "fermentation vessel" in which cellulose and starch are fermented by ruminal microflora [6]. Stopping an IVDMD reaction after 12 h provides an estimate of the rate of starch digestion, since this approximates the period of time contents normally stay in the rumen [29], and is an important indicator of its value as feed for ruminants. However, results from this method are not directly related to what would result from digestive mechanisms in monogastric organisms, such as humans (Homo sapiens).

The in vitro pepsin digestion assay is commonly used to assess sorghum digestibility [30] because of its ease of use. However, this assay mimics only part of the digestive system, i.e., the stomach, where partial digestion of protein occurs in an acidic environment and where no starch is digested. As with pepsin for protein digestion, in vitro starch digestion by α-amylase offers a similar partial solution, for protein digestion. Amylase digestion takes place in an alkaline environment, approximating that of the small intestine [10]. These two in vitro methods can be used to analyze digestion of protein or starch separately, but they do not address effects of interactions between the polymers in raw meal [31] or their biochemical interactions during and after cooking [26,32].

Compared to the separate pepsin and starch digestion assays described above, in vitro pancreatin digestion (also known as digestion by simulated intestinal fluid) is underutilized in studying sorghum digestibility. As reported in the literature, its main use is to monitor the digestibility of potential food allergens [33]. Pancreatin, produced by the pancreas, contains numerous enzymes, e.g., trypsin, chymotrypsin, lipase, amylase, colipase, and ribonuclease [34], thus providing the capacity to act on both protein and starch and other feed components in a single treatment. Making use of the multiple activities of pancreatin could give insight into the interrelationships of starch and protein and their interactive effects on grain digestibility. Potential difficulties with the use of pancreatin include the fact that its composition is variable and its action is downstream of the stomach.

Although numerous methods have been devised to assess the food and feed value of sorghum grain, as described above, the use of these methodologies by breeders to improve digestibility has been ineffective to date. This shortcoming may be due to inherent variability in the methods themselves, as well as their ineffectiveness in identifying the fundamental basis of variability in digestibility of the grain. To elucidate the basis of the latter problem, we applied multiple in vitro methods to assess protein and starch digestibility.

In the first set of experiments, disappearance of dry matter by IVDMD, digestion of protein by pepsin and digestion of starch by α-amylase were measured on 18 non-isogenic and isogenic waxy and non-waxy sorghum varieties and, for comparison, two maize varieties. Results from these experiments define the range of variation within these 18 seedlots. In the second set of experiments, selected waxy and non-waxy lines were further analyzed using principal component analysis (PCA) to identify the factors that account for total variance within populations of the selected lines. In the third set of experiments, selected waxy and non-waxy lines were digested separately in vitro in a time-dependent manner by performing pancreatin assays in parallel with pepsin and α-amylase assays. By comparing the pancreatin results with the pepsin and α-amylase results, we discerned differences in the digestibility of starch and specific proteins both between and within the waxy and non-waxy grains. In analyzing these data we identified a set of narrowly defined targets that warrant further study in the application of molecular genetics for improving the digestibility of sorghum grain.

2. Materials and methods

2.1. Materials

The sources of sorghum and maize grain samples used in this study are listed in Table 1. Unless otherwise indicated below, the samples were initially grown in Lincoln NE and selected to represent a broad range in digestibility based on 12-h IVDMD. P898012 seed was obtained from Pioneer Hybrid; 296B seed was obtained from Dr. Ian Godwin, University of Queensland, Australia. Grain of the latter two varieties used in experiments described in this paper was grown in the greenhouse at UC Berkeley. Near-isogenic maize lines, gifts of Dr. Jay Hollick, were also grown in the greenhouse at UC Berkeley. Samples of mature dried sorghum and maize grain

Table 1
Seed sample of 18 sorghum and two maize lines of waxy and non-waxy phenotype.

Inbred	Species	Seed source	GBSS1	Starch type	High-tannin testa	Origin of line
296B	Sorghum	UQ	+	Non-waxy	Absent	Indian food grade variety
B35	Sorghum	USDA	+	Non-waxy	Absent	Tx642 [55]
Tx7078	Sorghum	USDA	+	Non-waxy	Absent	[56]
IA19	Sorghum	USDA	+	Non-waxy	Present	[57]
RTx430	Sorghum	USDA	+	Non-waxy	Absent	[58]
Wheatland	Sorghum	USDA	+	Non-waxy	Absent	[59]
P898012	Sorghum	Pioneer	+	Non-waxy	Present	Purdue Experimental line
KS51	Sorghum	USDA	+	Non-waxy	Absent	[53]
BTx630	Sorghum	USDA	−	Waxy	Absent	[60]
N38	Sorghum	USDA	−	Waxy	Absent	[61]
BTxAGR-1	Sorghum	USDA	(+)	Waxy	Absent	[62]
KS48	Sorghum	USDA	−	Waxy	Absent	[53]
Tx2907	Sorghum	USDA	−	Waxy	Absent	[63]
B9307	Sorghum	USDA	(+)	Waxy	Absent	TAMU Experimental waxy line
05-3768-2N	Sorghum	USDA	+	Non-waxy	Absent	USDA Experimental line – near isogenic non-waxy
05-3768-3W	Sorghum	USDA	−	Waxy	Absent	USDA Experimental line – near isogenic waxy
05-3771-4N	Sorghum	USDA	+	Non-waxy	Absent	USDA Experimental line – near isogenic non-waxy
05-3771-1W	Sorghum	USDA	−	Waxy	Absent	USDA Experimental line – near isogenic waxy
B73	Maize	UCB	+	Non-waxy	Absent	[64]
T6-9WX	Maize	UCB	−	Waxy	Absent	UCB Experimental line near isogenic waxy B73

GBSS1 = granule bound starch synthase 1; + = active; (+) = inactive; − = absent. USDA = USDA-ARS, Lincoln, NE; UQ = University of Queensland, Brisbane, Australia; UCB = University of California, Berkeley, CA; TAMU = Texas A&M University, College Station, TX.

were ground in a Wiley Mill through a 40-mesh screen. Ground meal was stored in sealed conical tubes at ambient temperature for short-term use, at 4 °C for long-term use.

Pepsin (porcine stomach mucosa, P-7000), pancreatin (porcine pancreas, P-7545), and two types of α-amylases, bacterial (A-3403, Type XIIA) and porcine pancreas (A-3176, Type VI-B), were purchased from Sigma–Aldrich (St. Louis, MO).

Antibodies used in this study were obtained from several sources. Mono-specific antibodies against 22 kDa α-zein (B) and 27 kDa γ-zein were from Dr. R. Jung, Pioneer Hi-Bred (Johnston, IA). Polyclonal antibodies were as follows: α-zein from Dr. B.A. Larkins (University of Arizona, Tucson, AZ); β-kafirin from Dr. B.R. Hamaker (Purdue University, West Lafayette, IN); waxy protein from Dr. S.R. Wessler (University of Georgia, Athens, GA).

2.2. Methods

2.2.1. Determination of reducing sugar, total starch, amylose and amylopectin

Reducing sugar concentration was measured with dinitrosalicylic acid [35] using glucose as standard. Total starch content was measured using a Starch Assay Kit – HK [SA-20] (Sigma–Aldrich, St. Louis, MO). The ratio of amylose and amylopectin was determined using a Megazyme Amylose/Amylopectin Assay Kit (Megazyme, Bray, Ireland).

2.2.2. In vitro dry matter digestion

A 12 h in vitro dry matter digestion with rumen fluid inoculum obtained from a ruminally fistulated steer was as previously described [29].

2.2.3. In vitro starch digestion rate

Two hundred mg of meal from each variety was suspended in 5 mL of ddH$_2$O in 50-mL conical polypropylene tubes and incubated in a 37 °C water bath for 20 min with occasional mixing. Twenty-five mL of an enzyme solution (porcine pancreatic α-amylase [Type VI-B], 10 units/mL in 1 mM Na glycerophosphate–HCl, pH 6.9 buffer containing 25 mM NaCl, 5 mM CaCl$_2$) was added to each tube. Mixture was incubated at 37 °C for 180 min with mixing every 10 min. At the indicated times: 0, 10, 20, 40, 60, 90, 120 and 180 min, a 1-mL aliquot of reaction mixture was removed and added to 1.5 mL microfuge tubes containing 0.1 mL 2 N H$_2$SO$_4$ to stop the reaction. After centrifugation at 14,000 rpm for 10 min in a table-top centrifuge (Eppendorf, 5417C), the supernatant fraction was removed and saved for reducing sugar determination (Section 2.2.1). Increase in reducing sugar over time was used to estimate the in vitro rate of starch digestion.

2.2.4. Protein content

Nitrogen content, N × 6.25 [36], of sorghum samples (3 × 5–6 mg of meal) was quantified by Dumas combustion method (NC-2100 Soil, CE Instruments). Acetanilide was used as standard.

2.2.5. Protein digestibility

Total protein of control and residual protein of 2-h pepsin-digested samples was quantified based on total nitrogen measured using the Dumas combustion method, as described above. The factor, N × 6.25 [36], was used to calculate protein N. Protein digestibility was calculated as the difference between total protein and residual protein after 2 h pepsin digestion, divided by total protein; results were expressed as a percentage.

2.2.6. In vitro rate of protein digestion – pepsin

The in vitro protein digestion assay with pepsin was modified from Aboubacar et al. [18]. Sorghum meal of each cultivar was weighed into 6× 1.5-mL microfuge tubes and labeled as control, 0, 30, 90, 120, and 180 min. One mL pepsin solution (Sigma P-7000, activity: 662 units/mg protein) containing 20 mg of pepsin in 0.1 M KH$_2$PO$_4$, pH 2.0, was added to each. Suspensions were vortexed, and the samples shaken horizontally in a microfuge rack at 37 °C in an incubator–shaker at 150 rpm. At indicated times sample tubes were removed and the reaction was stopped with 100 μL of 2 N NaOH. Tubes were centrifuged at 14,000 rpm for 10 min at RT; supernatant fractions were discarded. Pellets were resuspended in 1 mL 0.1 M KPi buffer, pH 7.0, mixed and centrifuged; supernatant fractions were discarded and pellets washed with ddH$_2$O and centrifuged as described above. Pellets were saved for protein extraction. Protein was extracted from undigested samples for comparison. Protein was extracted from residues undigested by pepsin using 0.5 mL 0.0125 M Borate–1% SDS–2% 2-ME buffer, pH 10 for 1 h at RT with continuous shaking [15,18,37,27]. Suspensions were centrifuged 10 min at 14,000 rpm. Supernatant fractions were saved for protein determination by NI Protein Assay or by SDS-PAGE analysis (Section 2.3.1).

Non-Interference Protein Assay (GBiosciences, St. Louis, MO) was used to determine the concentration of extracted protein. Interfering substances were removed by precipitating protein with Universal Protein Precipitation Agent (UPPAI and UPPAII), according to manufacturer's instructions. The amount of copper ion not binding to peptide backbone of solubilized protein was determined colorimetrically at 480 nm; BSA was used as standard.

The decreased amounts of protein extracted over time were used to estimate rates of protein digestion. The amount of protein extracted from each sample was plotted across all time points with the linear portion of the plot visually identified as occurring from 30 to 120 min; rates were calculated for the linear period.

2.2.7. In vitro rate of protein digestion – pancreatin

Fifty mg of sorghum meal from each variety was placed in 1.5 mL microfuge tubes labeled as control, 0, 30, 90, 120, and 180 min. Each sample was mixed with 1.0 mL of simulated intestinal fluid, 1% porcine pancreatin (w/v), 48.9 mM monobasic potassium phosphate and 38 mM NaOH; pH was adjusted to 7.5 with NaOH [38]. Reaction mixtures were incubated at 37 °C with continuous shaking for 180 min. At indicated times, the reaction was stopped with 1/10 volume of 100 mM phenylmethylsulfonyl fluoride (PMSF). After termination, reaction mixtures were centrifuged at 14,000 rpm for 10 min at RT. Supernatant fractions were removed and saved for reducing sugar determination (Section 2.2.1). After washing twice with 1 mL ddH$_2$O, pellets were extracted for protein and used for protein determination and calculation of digestion rates as described in Section 2.2.6.

2.2.8. In vitro rate of starch digestion – pancreatin

The procedure for determining *in vitro* starch digestion rate by pancreatin was as described (Section 2.2.7), except clear supernatant fractions were used for measuring reducing sugar; time points were 0, 30, 90, 120, and 180 min. Calculations of rates were based on apparent linear portions of digestion plots, which occurred between 3 and 120 min.

2.2.9. In vitro rate of starch digestion – bacterial amylase

The determination of *in vitro* rates of starch digestion by bacterial amylase was as described above (Section 2.2.3) except α-amylase was from *Bacillus licheniformis* (Type XII-A); time points were 0, 10, 20, 40, 60, 90, 120, 150, and 180 min. Rate calculations were based on the apparent linear portion of the digestion plots, which occurred between 20 and 120 min.

2.3. SDS-PAGE patterns of in vitro protein digestion

2.3.1. NuPAGE analysis of extracted kafirins and glutelins

Protein samples extracted following *in vitro* pepsin digestion (Section 2.2.6) were analyzed by SDS-PAGE. NuPAGE Novex Bis–Tris gels (Invitrogen, Carlsbad, CA) or Criterion XT Bis–Tris gel (Bio-Rad, Hercules, CA) provided better separation and resolution of small- to medium-sized proteins due to a neutral pH environment that minimizes protein modifications [39,27]. Aliquots (usually 100 μL of protein extract) were precipitated with 5 vol of acetone at −20 °C overnight. Pellets recovered after centrifugation were redissolved in 50 μL 1× NuPAGE sample buffer supplemented with 0.1% 2-ME, boiled for 5 min, clarified by quick centrifugation and then subjected to electrophoresis on a 12% Bis–Tris NuPAGE or Criterion XT Bis–Tris gel with MOPS buffer for 1 h 20 min at 150 V at RT. Gels were stained with colloidal Coomassie G-250 overnight and de-stained in several changes of ddH$_2$O.

2.3.2. Extraction, SDS/PAGE and western analysis of granule bound starch synthase

GBSS1 was obtained from the remaining residues after kafirin extraction (Section 2.3.1) by boiling in excess Borate–SDS–ME buffer [27]. Separation of extracted proteins by SDS/PAGE in 10–20% Tris–Glycine Criterion gels and western blot analysis with antibody against waxy protein were as described [27].

2.3.3. Quantification of undigested protein separated on PAGE

Protein gels were scanned with a UMAX PowerLook 1100 scanner (UMAX.com) in Photoshop. Gel images in tiff format were transferred and volume (intensity × area) of kafirin bands quantified using the Quantity One program (Bio-Rad, Hercules, CA). Higher band volumes indicated more protein remaining and thus protein digestibility as being lower.

2.3.4. Pattern of total seed protein extract

Extraction of total seed protein was conducted under reducing conditions with an SDS-containing buffer. Fifty mg samples of sorghum and maize meals were extracted with 1 mL 1× Extraction Buffer (50 mM Tris–HCl, pH 6.8; 2% SDS; 100 mM DTT) in 1.5 mL microfuge tubes. Sample tubes were capped, vortexed briefly and immediately heated at 100 °C for 4 min in a heating block, vortexed 10 s, reheated for 4 min and then vortexed 15 s to extract protein. Samples were centrifuged for 10 min at 14,000 rpm at RT. Supernatant fractions were transferred to new tubes and volumes noted. Extracted samples were stored at −20 °C. Protein content was determined by Non-Interference Protein Assay (Section 2.2.6).

2.4. Western blots

Equal volumes (5 μL) of undigested extracts from *in vitro* pepsin digestion experiments were used to generate the data in Fig. 5. Equal amounts (20 μg) of total protein extracted from each line (see Section 2.3.4) were used for the data in Fig. 4. Protein samples were separated on 12% Bis–Tris gels using MOPS buffer (Section 2.3.1). Protein was transferred to a nitrocellulose membrane at 70 V for 80 min at 4 °C. Primary antibody (as specified in legends of Figs. 4 and 5) was used at 3.2 μL/20 mL 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 color development procedure with multiple antibodies was as described [40] with modifications: using 5% powdered milk instead of BSA and omitting the chemiluminescent detection step. All peroxidase substrates were obtained from Vector Laboratories (Burlingame, CA). Antibody-color substrates used were: anti α-zein Ab – SG (dark grey), anti 27 kDa γ-zein Ab – DAB (brown); anti β-kafirin Ab – TMB (purple) and reprobing with anti γ-kafirin Ab – SG (dark grey).

2.5. Statistical analysis

Data analyses were generated using SAS/STAT software, Version 9.2 of the SAS System for Windows© 2002–2008, SAS Institute Inc.[1] Least square means and standard errors of traits measured for each line and *F*-tests for significant differences among lines were calculated using the MIXED procedure. An estimate of precision of each laboratory procedure was made by dividing the median mean value for each trait by its standard error and multiplying by 100. Eigenvalues of the covariance matrix, which describe the proportion of total variance attributable to their respective principal components, and the corresponding Eigenvectors of the principal
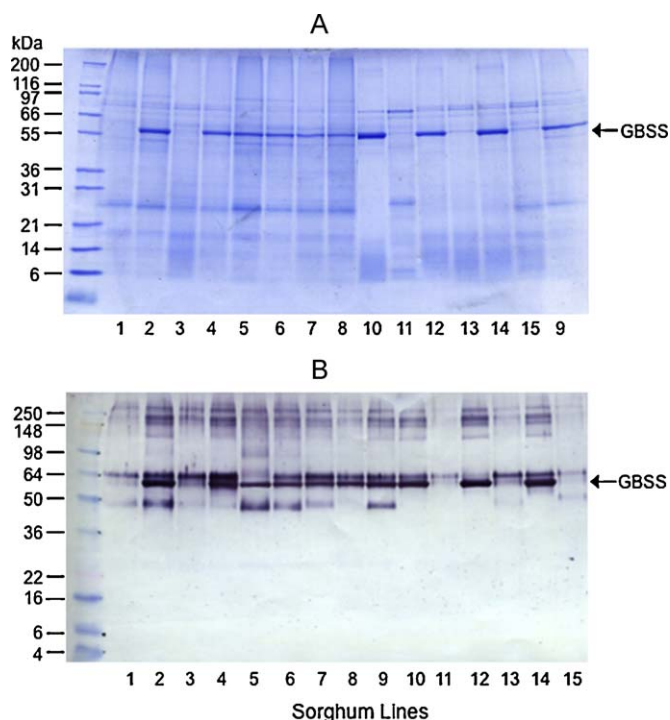

Fig. 1. The presence or absence of granule bound starch synthase (GBSS1) in non-waxy and waxy phenotypes of sorghum and maize in Borate–SDS–ME boiled extracts. (A) Stained protein gel; (B) western blot using waxy protein antiserum. Lanes 1, 3, 11, and 13 are waxy sorghum varieties, KS48, N38, 05-3768-3WX, and 05-3771-1WX (respectively); lane 15 is waxy maize line, T6-9WX; lanes 2, 4–10, and 12 are non-waxy sorghum varieties, KS51, B35, RTx430, 296B, Tx7078, IA19, P898012, 05-3768-2N, and 05-3771-4N, respectively; lane 14 is non-waxy maize line, B73.

components, which describe the weight attributable to the measured traits for those principal components, were calculated using the PRINCOMP procedure.

3. Results and discussion

3.1. Composition and common digestion characteristics of grain from 18 sorghum and two maize varieties

The contents of total starch, amylose and total protein together with digestibility characteristics (in terms of dry weight, protein and starch) were determined for 18 sorghum and two maize varieties (Table 2). Waxy and non-waxy classification of these varieties was based on the absence or presence, respectively, of granule bound starch synthase (GBSS1) in grain extracts as revealed by western blot analysis (Fig. 1 and Table 1, column 4). The presence or absence of GBSS1 in the three lines not shown in Fig. 1 was reported elsewhere [8]. GBSS1 functions in the biosynthesis of amylose [9]. A major isozyme is located in the endosperm and a minor form in the pericarp [41]. The absence of GBSS1, or its inactivity, in sorghum grain, i.e., BTxAGR1 and B9307 (Table 1, column 4), is associated with starch containing a low proportion of amylose (Table 2, column 3); resulting grain stains differently with iodine [42]. *F*-tests for differences among lines were significant for all traits except *in vitro* rate of starch digestion (IVRSD pancreatin) which was not testable.

3.1.1. In vitro dry matter disappearance (IVDMD)
Grain samples were selected to exhibit a range of 12-h IVDMD values, from a low of 19.3% to a high of 33.0% (Table 2, column 4). Precision or repeatability of the 12-h IVDMD procedure was high with standard errors being 8% of the line median. The low value

belongs to a non-waxy sorghum, IA19, which possesses a testa layer with a high concentration of tannins, and the high value to a waxy sorghum variety, KS48, which contains no tannin. Interestingly, P898012, which has a testa layer with tannin, surprisingly has 12-h IVDMD values comparable to several non-tannin containing sorghums. IA19 had similar amounts of starch, compared to P898012 (63.5% vs. 61.4%), but its starch digestion rate was much lower as indicated by either 12-h IVDMD (19.3% vs. 23.0%) or *in vitro* starch digestion rate (IVSDR) (0.0022 mg glucose/min/mL vs. 0.0067 mg glucose/min/mL) (Table 2, columns 4 and 5). Protein digestion was also dissimilar (44.8% vs. 58.9%) [Table 2, column 7 (PD)]. The lower digestion rates in IA19 are consistent with the binding of carbohydrates and proteins to tannin with the resulting inhibitory effect on α-amylase [25,43]. Why these inhibitory effects were not observed for P898012 is not obvious, but results clearly identify this line as a unique testa-layer-containing line with digestibility parameters equivalent to non-testa-containing counterparts.

3.1.2. IVSDR
Results of a series of time-course, starch digestions with pancreatic α-amylase are shown in Table 2, column 5 (IVSDR). Waxy varieties generally had higher starch digestion rates than their non-waxy counterparts. Among sorghum lines, N38, a waxy variety, had the highest IVSDR (0.0102 mg glucose/min/mL) and IA19, a non-waxy sorghum with a tannin-containing testa, had the lowest rate (0.0022 mg glucose/min/mL). Standard error was 11% of the line median. IVSDR and 12-h IVDMD essentially measure the same parameter – the rate of starch digestion. Similar IVSDR results and 12-h IVDMD results were, therefore, expected and observed.

3.1.3. Percent protein digestibility (%PD) by pepsin
Values for percent protein digestibility (%PD) ranged from 34.6% to 71.5% with the highest values associated with the two maize lines (waxy and non-waxy) that were included as references (Table 2, column 7). The highest %PD value (62.5%) for sorghum was associated with a waxy, non-tannin variety, KS48, followed by a value of 58.9% for the non-waxy, tannin-containing variety, P898012. The lowest value (34.6%) was associated with another non-waxy sorghum, Wheatland, with the next highest value (38.4%) actually belonging to a waxy line with no GBSS1, BTx630 (Table 1). The wide range in %PD (34.6–71.5%) suggests that this set of lines is quite diverse. The precision of the PD assay was very high with the standard error only 5% of the line median.

It is again noteworthy that the %PD of P898012, a non-waxy testa-containing line, ranked second only to the waxy, non-tannin line, KS48. Data in Table 2 indicate that both the P898012 and KS48 lines had very similar protein content (11.7% vs. 12.3%, respectively) and %PD (58.9% vs. 62.5%, respectively). This finding contradicts the widely held view regarding the effect of tannins on sorghum digestibility. Our results indicate that P898012 is a true outlier with respect to other scientific studies regarding the effects of tannin on sorghum digestibility.

3.2. Digestion characteristics of thirteen sorghum grain samples using pepsin, pancreatin and bacterial enzymes

To obtain further insight into the digestibility pattern of protein and starch, both individually and in association with one another, thirteen sorghum lines – six waxy (BTx630, N38, BTxARG1, KS48, Tx2907, and B9307) and seven non-waxy (296B, B35, Tx7078, IA19, RTx430, Wheatland, and KS51) with varying digestibility scores – were selected for further analyses using time-course, *in vitro* digestion assays with pepsin, α-amylase, and pancreatin (Table 2, columns 8–11). This subset is representative of typical elite sorghum lines. Since it had been identified as being unique, in terms

Table 2
Characterization of 18 sorghum and two maize lines of waxy and non-waxy phenotype.

Line	Total starch (%)	Amylose (%)	12-h IVDMD[a] (%)	IVSDR[a] (mg glucose/min/mL × 10^{-3})	Protein (%)	PD[a] (%)	IVRPD[a] pepsin (%/min/mL)	IVRPD[a] pancreatin (%/min/mL)	IVRSD[a] pancreatin[b] (mg glucose/min/mL × 10^{-3})	IVRSD[a] bacterial α-amylase (mg glucose/min/mL × 10^{-3})
Sorghum										
296B	70.6	29.0	27.4	5.6	13.0	52.7	0.46	0.55	2.6	1.2
B35	72.9	25.1	23.6	6.0	12.3	47.5	0.57	0.54	2.3	1.1
Tx7078	75.0	26.7	31.1	5.9	11.4	42.4	0.60	0.61	2.4	1.2
IA19	63.5	28.5	19.3	2.2	15.0	44.8	0.61	0.22	2.7	0.5
RTx430	56.0	28.1	24.3	5.0	12.5	42.1	0.65	0.48	2.4	1.4
Wheatland	85.0			5.5	11.4	34.6	0.13	0.32	4.2	4.3
P898012	61.4	28.2	23.0	6.7	11.7	58.9				
KS51	80.5	22.5	22.1	6.4	13.1	56.2	0.52	0.61	2.7	1.4
BTx630	68.2	5.7	26.3	7.2	10.1	38.4	0.59	0.55	6.8	5.0
N38	68.4	8.9	32.6	10.2	11.5	53.0	0.68	0.62	5.3	2.4
BTxAGR-1	80.6	11.8	22.2	5.9	11.3	54.0	0.67	0.55	6.5	2.9
KS48	47.9	11.5	33.0	9.1	12.3	62.5	0.65	0.68	4.8	2.1
Tx2907	77.9			7.6	11.8	52.9	0.41	0.66	6.7	3.8
B9307	72.9			6.1	12.0	48.6	0.25	0.29	6.2	4.1
05-3768-2N	76.3	26.3	23.6	5.8	12.3	51.7				
05-3768-3W	70.3	7.5	22.7	9.2	12.4	56.7				
05-3771-4N	74.6	31.9	23.5	6.2	14.7	46.6				
05-3771-1W	80.9	9.6	21.7	9.1	13.3	50.8				
Maize										
B73	80.8	39.2	23.5	7.6	11.3	70.6				
T6-9WX	66.6	10.1	26.0	12.0	10.0	71.5				
Standard error[c]	5.3	5.0	2.0	0.7	0.4	2.6	0.06	0.10		0.7
Range for sorghum	47.9–80.9	5.7–31.9	19.3–33.0	2.2–10.2	10.1–15.0	34.6–62.5	0.13–0.68	0.22–0.68	2.4–6.8	0.5–5.0
Precision (SE/median × 100)	8	27	8	11	3	5	14	22		25

[a] IVDMD = in vitro dry matter digestibility; IVSDR = in vitro rate of starch digestion by pancreatic α-amylase; PD = protein digestion; IVRPD = in vitro rate of protein digestion by pepsin or pancreatin; IVRSD = in vitro rate of starch digestion by pancreatin or bacterial α-amylase.

[b] Least square means and standard errors for IVRSD pancreatin were not estimable due to lack of replication. Observed values are shown.

[c] Standard errors shown are the most conservative (largest) for any least square mean in the column.

of tannin and digestion characteristics, P898012 was not included in order to restrict inference to lines representative of most elite sorghums.

3.2.1. In vitro rate of protein digestion (IVRPD) by pepsin

Values of IVRPD ranged from 0.13 to 0.68%/min/mL with similar distribution of values among waxy and non-waxy sorghums [Table 2, column 8 (IVRPD pepsin)]. The non-waxy line with the highest IVRPD (0.65%/min/mL) was RTx430; the non-waxy line with the lowest IVRPD was Wheatland (0.13%/min/mL). The waxy line with highest IVRPD by pepsin was N38 (0.68%/min/mL); the waxy line with the lowest IVRPD was B9307 (0.25%/min/mL). Precision was relatively moderate, with the standard errors being 14% of the line median.

3.2.2. In vitro rate of protein digestion (IVRPD) by pancreatin

Values of IVRPD ranged from 0.22 to 0.68%/min/mL with similar distribution of values among waxy and non-waxy lines [Table 2, column 9 (IVRPD pancreatin)]. The non-waxy line with highest IVRPD by pancreatin was Tx7078 (0.61%/min/mL); the non-waxy line with the lowest IVRPD was IA19 (0.22%/min/mL). The waxy line with the highest IVRPD by pancreatin was KS48 (0.68%/min/mL); the waxy line with the lowest IVRPD by pancreatin was B9307 (0.29%/min/mL). Precision was low, with standard errors being 22% of the line median. It was surprising that the multiple functions of pancreatin did not contribute to greater discrimination in protein digestion among waxy and non-waxy starch or among samples. This may be due, in part, to the reduced precision of this parameter relative to other digestion analyses or to a general masking of the effects of different starches on digestion of storage proteins (i.e., α-, γ-, and β-kafirins) when measuring total protein. Interactions of starch types with digestion of storage proteins will be discussed further in Sections 3.5 and 3.6.

3.2.3. In vitro rate of starch digestion (IVRSD) by pancreatin

Values of IVRSD ranged from 2.4 to 6.8 mg glucose/min/mL with starch digestion rates of meal from waxy sorghums generally being double those of non-waxy sorghums [Table 2, column 10 (IVRSD)]. The waxy line with the highest IVRSD by pancreatin was BTx630 (6.8 mg glucose/min/mL); the waxy line with the lowest IVRSD was KS48 (4.8 mg glucose/min/mL). The non-waxy line with the highest IVRSD by pancreatin was Wheatland (4.2 mg glucose/min/mL); the non-waxy line with the lowest IVRSD was B35 (2.3 mg glucose/min/mL). Precision was not estimable due to lack of replication. Unlike IVRPD by pancreatin, this procedure appeared to discriminate between lines with waxy and non-waxy starches; relative differences were larger than those for IVSDR (Table 2, column 5). The multiple functions of pancreatin apparently contributed to greater discrimination in starch digestion. Although experimental precision was not estimable in this analysis, it seems reasonable to expect more apparent effects of the multiple pancreatin functions on starch digestion, compared to protein digestion, since starch makes up a much larger proportion of the grain.

3.2.4. In vitro rate of starch digestion (IVRSD) by a bacterial α-amylase

Values for IVRSD by bacterial amylase ranged from 0.5 to 5.0 mg glucose/min/mL with digestion rates of waxy samples again generally being double those of non-waxy sorghums [Table 2, column 11 (IVRSD α-amylase)]. Compared to IVRSD, pancreatin [Table 2, column 10 (IVRSD pancreatin)] that uses porcine pancreatic α-amylase, digestion rates using B. licheniformis α-amylase were much slower, roughly halved. Precision was the lowest of any analyses conducted, with the standard error being 25% of the line median.

Table 3A
Eigenvalues of the covariance matrix using ten sorghum lines and ten variables.

	Eigenvalue	Difference	Proportion	Cumulative
Principal component 1	117.08	26.53	0.43	0.43
Principal component 2	90.55	43.58	0.33	0.77
Principal component 3	46.97	31.54	0.17	0.94
Principal component 4	15.43	15.07	0.06	1.00
Principal component 5	0.36	0.36	0.00	1.00
Principal component 6	0.00	0.00	0.00	1.00
Principal component 7	0.00	0.00	0.00	1.00
Principal component 8	0.00	0.00	0.00	1.00
Principal component 9	0.00	0.00	0.00	1.00
Principal component 10	0.00		0.00	1.00

Table 3B
Eigenvectors of the principal components using ten sorghum lines and ten variables.

	Principal component 1	Principal component 2	Principal component 3	Principal component 4
Starch	0.84	0.51	0.08	0.16
Amylose	0.41	−0.79	0.39	0.20
12-h IVDMD	−0.25	0.11	0.02	0.95
IVRSD	0.00	0.00	0.00	0.00
Protein	0.01	−0.09	0.10	−0.13
Protein digestibility	−0.24	0.30	0.91	−0.11
IVRPD by pepsin	0.00	0.00	0.00	0.00
IVRPD by pancreatin	0.00	0.00	0.00	0.02
IVRSD by pancreatin	0.00	0.00	0.00	0.00
IVRSD by bacterial amylase	0.00	0.00	0.00	0.00

3.3. Principal component analysis

Considerable descriptive data regarding sorghum grain digestibility were collected (Table 2). At first glance, the simple question "which analyses are best?" begs to be answered. Most of the different analyses were designed to be "best" for a specific use. A question to which the answer has much broader application is "which analyses best describe variation in sorghum digestibility"? Principal component analysis (PCA) provides a tool that allows an answer to this question and provides mechanisms to describe relationships between starch and protein in the digestion of sorghum. This tool led us to the identification of factors that will be the focus of future efforts to perform targeted changes to positively affect sorghum digestibility.

Initial PCA included data from the ten lines that had data for all ten variables (Table 2). Results of the initial PCA revealed that 94% of the variation among the lines was attributable to the first three principal components, nearly 100% (99.86% before rounding) to the first four principal components (Tables 3A and 3B). Traits contributing most heavily to variation were starch content, starch type, 12-h IVDMD, protein content, and protein digestibility, while those not making significant contributions were in vitro rates of protein and starch digestion by pepsin, pancreatin and a bacterial enzyme.

By excluding traits that did not add to the variation, it was possible to utilize samples from a broader set of sorghum lines. The second PCA included 14 lines and six variables; results are in Tables 4A and 4B. Again, 94% of variation among lines was attributable to the first three principal components, nearly 100% (99.75% before rounding) to the first four principal components. Traits contributing most heavily to those principal components were, again, starch content, starch type, 12-h IVDMD, protein content, and protein digestibility. Data represented in a three-dimensional graph show relatively uniform distribution of the first three principal components for these 14 lines (Fig. 2).

With the exception of % amylose, all other traits contributing strongly to the first four principal components had estimates of

Table 4A
Eigenvalues of the covariance matrix using 14 sorghum lines and six variables.

	Eigenvalue	Difference	Proportion	Cumulative
Principal component 1	105.07	14.38	0.43	0.43
Principal component 2	90.70	55.68	0.37	0.80
Principal component 3	35.01	21.54	0.14	0.94
Principal component 4	13.48	12.87	0.06	0.98
Principal component 5	0.61	0.61	0.00	1.00
Principal component 6	0.00		0.00	1.00

Table 4B
Eigenvectors of the principal components using 14 sorghum lines and six variables.

	Principal component 1	Principal component 2	Principal component 3	Principal component 4
Starch	0.45	0.87	0.06	0.22
Amylose	0.81	−0.46	0.33	0.10
12-h IVDMD	−0.19	−0.15	0.03	0.95
IVRSD	0.00	0.00	0.00	0.00
Protein	0.06	−0.03	0.08	−0.17
Protein digestibility	−0.32	0.12	0.94	−0.06
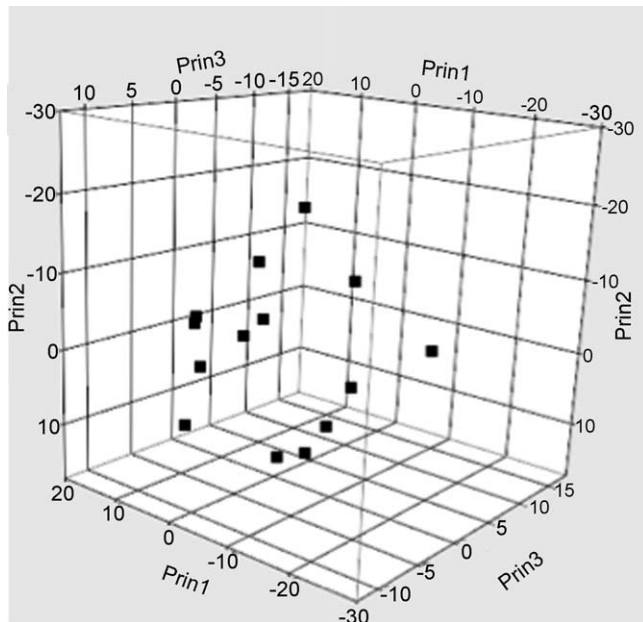
Fig. 2. Plot of principal components of 14 sorghum lines.

precision associated with their measurement below 10%. When different digestion assays measure, in a biological or chemical manner, essentially the same parameter (*e.g.*, 12-h IVDMD, IVSDR by pancreatic α-amylase, IVSDR by bacterial α-amylase), it is not surprising that the most repeatable assay is the one contributing most strongly to the principal components. This is fortunate in practical, as well as biological, terms because assays with the lowest errors tended to be the simpler technical assays to conduct in the laboratory.

Given that the first three principal components accounted for 94% of the variation in this population of samples and given that starch type (amylose content), starch content, 12-h IVDMD, and protein digestibility contributed most strongly to these principal components, a strong argument can be made that, in the practice of evaluating or screening sorghum for digestibility, emphasis should be placed on these traits. Since grain samples used in this study were initially selected for wide variation in 12-h IVDMD, it is logical that this trait would contribute strongly to the principal components. Caution should, therefore, be exercised in including the 12-h IVDMD in the set of traits to be used for selection.

It is obvious that the type of starch (waxy or non-waxy), amount of starch, and protein digestibility are primary controlling factors of sorghum digestibility. Presently most researchers and end users prudently segregate sorghum grain by starch type. After performing that segregation, our results indicate that, at least in the broad sense of dealing with total variation, focus should be placed on increasing starch content and protein digestibility.

3.4. One-dimensional gel patterns of total protein in various sorghum cultivars

Our laboratories have had long-term interest in the redox-regulated mobilization (or digestibility) of disulfide storage proteins in cereals. It is well documented that the protein digestibility problem in sorghum is linked to the disulfide status of its storage proteins [19,44]. Protein digestibility is expressed as a percentage, calculated as the difference between total protein and residual protein after a 2 h pepsin digestion, divided by total protein. Residual protein is known to be composed mostly of kafirin, especially α-kafirin, the most abundant and easily digestible kafirin, that is located in the central region of the protein body and is surrounded by the cysteine-rich β- and γ-kafirins on the periphery. Because of their ability to form disulfide-bonded structures that resist pepsin digestion, β- and γ-kafirins have been proposed to impede proteolysis of the more abundant α-kafirins – one of the main causes of indigestibility in sorghum [15,16,45,14,46]. Thus, the amounts of β- and γ-kafirins, relative to α-kafirin, reflect variation in protein body packaging and digestibility among sorghum varieties [47].

Total extractable grain protein of eight waxy (A) and ten non-waxy (B) sorghum lines, relative to maize (C) (listed in Table 2) was visualized in one-dimensional polyacrylamide gels (Fig. 3). Intensities of all protein bands were quantified and ratio values of high M_r protein (>40 kDa) to low M_r protein (<30 kDa) among waxy lines were calculated; they ranged from 0.443 to 1.134, with a median of 0.876 (std, 0.247; std err, 0.078; and variance 0.061). Ratio values among non-waxy lines ranged from 0.436 to 0.943 with a median, 0.595 (std, 0.146; std err, 0.042; and variance 0.021). Therefore, the proportion of high M_r protein to low M_r protein was 0.88–1.0 among waxy lines compared to 0.60–1.0 among non-waxy lines. These analyses showed that, among waxy lines, the proportion of high M_r protein was 0.88, which is higher than the 0.60 obtained for the non-waxy lines. Low M_r proteins are mostly different types of kafirins (Section 3.5). The remaining low and high M_r proteins are non-kafirins, not only consisting mostly of glutelins, but also including albumins and globulins as defined under the proposed new functional sorghum protein classification scheme [15,31]. This observation suggests that the higher M_r endosperm protein, likely making up the protein matrix, is more variable among waxy lines and is different in quantity from that in non-waxy lines. This is also indirectly supported by the results from pancreatin treatments (Section 3.2.3) that show protein affects starch digestibility more in non-waxy varieties than in their waxy counterparts. These differences in endosperm proteins may also be reflective of the genetic diversity of the sorghum varieties used in this study.

Kafirins, located in protein bodies, are subdivided into four types and are reported in the literature as: α- (23 and 25 kDa), β- (20 kDa), γ- (28 kDa) and δ- (10 kDa) kafirins; the abundance ratio of α:γ:β is 80:15:5. The δ-kafirin is a minor component, whose abundance has not been determined [48–50,15,16,51,46]. Western blot analyses with antibodies against α-zein, β-kafirin and γ-zein (Fig. 4) were used to show relative abundance of monomeric forms and occurrence of any polymeric forms of these three kafirin types among waxy (Fig. 4A–C) and non-waxy sorghum (Fig. 4D–F) lines. The antibodies, raised against zeins (maize prolamins), crossreact with the homologous sorghum kafirins [52]; their cross immunoreactivities
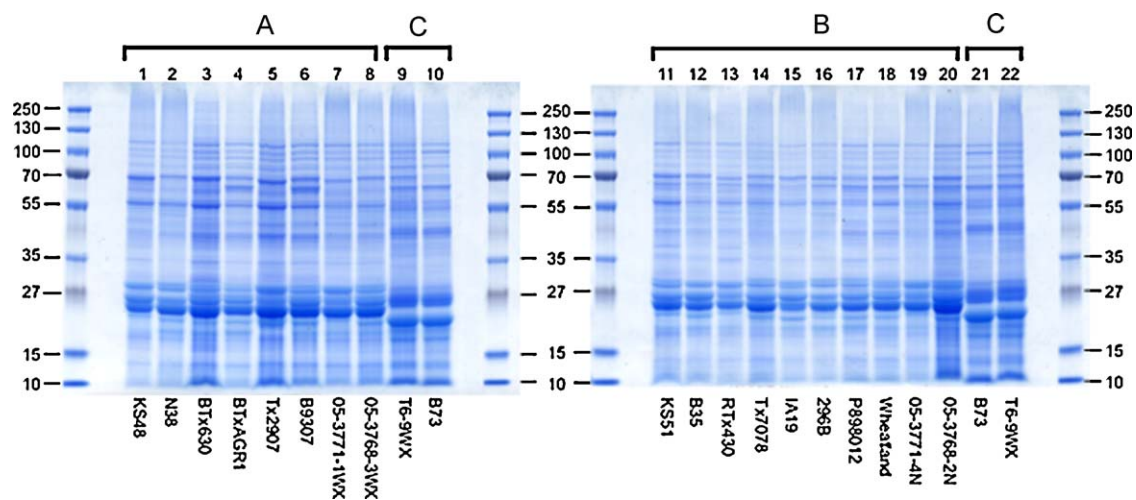
Fig. 3. One-dimensional gel pattern of total protein extract from eight waxy (A) and ten non-waxy (B) sorghum varieties with two reference maize lines (C). Equal amounts of protein (10 μg) from each cultivar were loaded in each lane. In (A) waxy sorghum, lanes 1–8 are KS48, N38, BTx630, BTxAGR1, Tx2907, B9307, 05-3771-1WX, and 05-3768-3WX, respectively; lane 9 is waxy maize line, T6-9WX; lane 10 is non-waxy maize line, B73. In (B) non-waxy sorghum, lanes 11–20, are KS51, B35, RTx430, Tx7078, IA19, 296B, P898012, Wheatland, 05-3771-4N, and 05-3768-2N, respectively; lane 21 is non-waxy maize line, B73; lane 22 is waxy maize line, T6-9WX. MW standards used were PageRuler Plus Prestained Protein Ladder (Fermentas Inc., Hanover, MD).
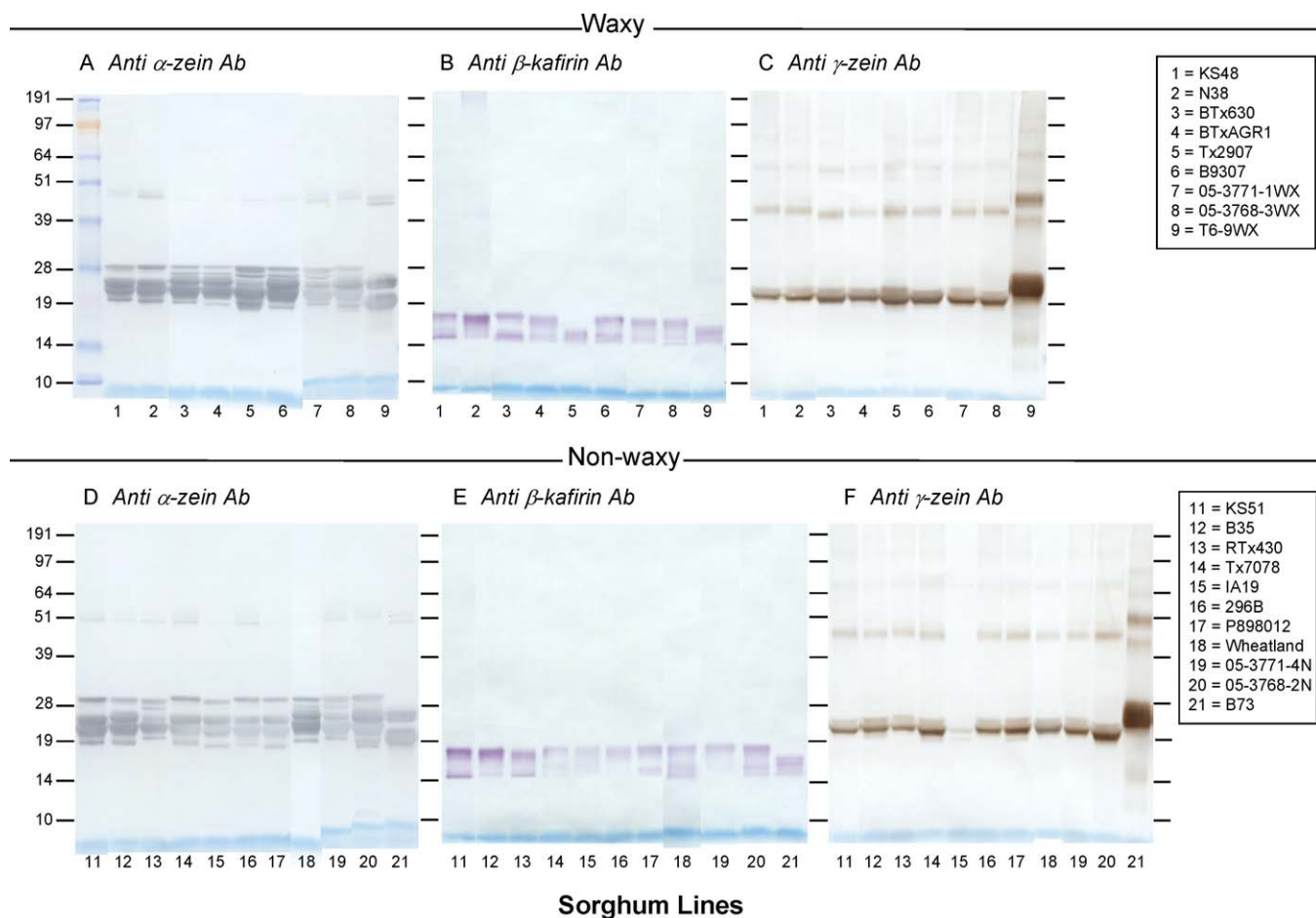

Fig. 4. Variations in the three types of kafirins visualized using western blot analysis on sorghum and maize lines. Eight waxy (A–C, top panel) and ten non-waxy (D–E, bottom panel) sorghum lines plus two maize lines are shown. Antibodies used were (A and D) anti α-zein antibody; (B, E) anti β-kafirin antibody; and (C and F) anti γ-zein antibody. In top panel (A–C) waxy sorghum, lanes 1–8 are KS48, N38, BTx630, BTxAGR1, Tx2907, B9307, 05-3771-1WX, and 05-3768-3WX, respectively; lane 9 is waxy maize line, T6-9WX. In bottom panel (D–F) non-waxy sorghum, lanes 11–20 are KS51, B35, RTx430, Tx7078, IA19, 296B, P898012, Wheatland, 05-3771-4N, and 05-3768-2N, respectively; lane 21 is non-waxy maize line, B73.

were also demonstrated by including maize samples (Fig. 4A–C, lane 9; C–F, lane 21).

The most abundant kafirin, the α-type, was shown to exist in multiple forms with M_r values ranging from 20 to 28 kDa, mostly monomers but with a trace amount detected as 46–50 kDa dimeric form (Fig. 4A and D). The major γ-kafirin with a M_r of 20 kDa overlapped with at least one α-kafirin, but the major form was smaller than the other two prevalent α-kafirins (25 and 28 kDa, respectively) and the maize γ-zein (M_r 27 kDa) (Fig. 4C, lane 9; F, lane 21). The 20 kDa M_r for γ-kafirin is different from that reported in the literature [16,14]. The γ-zein antibody also recognized several oligomeric kafirin forms of M_r 42, 60 and 72 kDa (Fig. 4C and F). Different forms of β-kafirins (18 and 15 kDa) were also detected in these samples (Fig. 4B and E) with their relative amounts varying based on the intensity of immunostaining. It is hard to rule out the existence of oligomeric forms of β-kafirin, but at the present level of protein loading, they were below the detection limit. Although not conclusive, our results suggest that variation in endosperm protein patterns, as well as in the packaging within protein bodies, is due to variation in kafirin types. These factors – variable endosperm protein patterns suggesting different protein networks and varying protein body compositions – appear to play a determining role in sorghum digestibility, as other studies have suggested [47].

3.5. Variation in digestion rate of different types of kafirins by pepsin

Compared to the %PD information provided by Dumas combustion, a gel-based method [18,37,27] provides more information on relative amounts and nature of the storage proteins that are not digested by pepsin. Proteins extracted from undigested residues could provide insight into the following questions. What type(s) of protein is (are) more resistant to pepsin digestion? Is the relative amount of each type of undigested protein related to indigestibility? Are the resistant proteins in less digestible sorghum lines present in forms (*i.e.*, oligomeric and polymeric) that might be more resistant to digestion?

A composite of NuPAGE gels and western blots (Fig. 5) demonstrates the patterns of protein digestion by pepsin of a pair of waxy and non-waxy sorghum lines with the same pedigree that differ in their digestibility (Texioca-63 × Short Kaura) [53,27]. The Borate–SDS–ME buffer is the buffer of choice to extract insoluble (storage) proteins that include kafirins and glutelins [15]. Under our experimental conditions (3 h period), a spectrum of proteins with M_r ranging from 15 to 200 kDa could be extracted from the insoluble proteins [see control (C) lanes (Fig. 5B)]. A majority of the high M_r protein bands ($M_r > 40$ kDa) were readily digested by pepsin as revealed by the diminished protein staining intensity relative to the undigested control over time (Fig. 5B). Various kafirins digested at different rates are shown in the accompanying protein gel quantification of KS48 (Fig. 5A) and KS51 (Fig. 5C). Western analyses with antibodies against different zeins and a kafirin demonstrated that a few high MW proteins were present as oligomeric kafirin forms (Fig. 5B–E). One protein of $M_r \sim 50$ kDa crossreacted with anti-22 kDa α-zein antibody (Fig. 5E, arrow). However, several protein bands at M_r 42, 60, and 72 kDa were recognized more strongly by γ-zein antibody than by α-zein antibody (cf. Fig. 5E to D, F, and G; bands of note designated by arrows); these bands were not recognized by the β-kafirin antibody (Fig. 5G). The remaining high M_r proteins showing no cross-reactivity are presumed to be glutelins because of the alkaline pH of the extraction solvent [15].

In contrast to high M_r oligomers, most proteins in the M_r range of 15–30 kDa were more resistant to pepsin digestion (Fig. 5A–C). Immunoreactivity revealed that these proteins were monomeric forms of different kafirins (Fig. 5D–G). This result contrasts with a previous report that α-kafirin is usually the protein remaining

after pepsin digestion because of being shielded from digestion by a protease-resistant, disulfide network of cysteine-rich β- and γ-kafirins [18]. Our results clearly show that multiple protein bands of M_r 18, 20, 25 and 28 kDa remain after 3 h of pepsin digestion (Fig. 5A). Another unexpected finding was revealed by immunoreactivity studies, namely that the most intense protein band observed during pepsin digestion was the 20 kDa γ-kafirin (Fig. 5D and F) – a band smaller than at least two of the α-kafirins at 25 and 28 kDa (Fig. 5E). γ-Kafirin was also shown to exist in several oligomeric forms (Fig. 5D and F; denoted by arrows). The 18 kDa β-kafirin was progressively digested (Fig. 5G).

In summary, the data in Fig. 5 reveal that different types of kafirins digest at different rates. The enhanced digestibility of waxy grain (KS48) relative to its non-waxy counterpart (KS51) is due to a combination of faster kafirin digestion rates, coupled with other more positive digestion parameters, such as less starch and higher starch digestion rates (Table 2). That the γ-kafirin in non-waxy KS51 is more resistant to digestion than in waxy KS48 is likely one of the reasons why KS51 is generally less digestible than KS48 (Fig. 5A–C). The observation that γ-kafirin is more resistant to digestion agrees with earlier work [20,16,37]. However, the strong reaction of the 20 kDa M_r γ-kafirin (plus a trace amount of α-kafirin), rather than α-kafirin alone, reveals that γ-kafirin is the abundant protein remaining after pepsin treatment. This is in stark contrast to earlier work [18]. We believe this finding gives new insight into the nature of kafirin packaging within the protein body.

3.6. Comparative pepsin digestion patterns of sorghum lines identified by PCA

As noted above, the PCA revealed that (i) type of starch, (ii) amount of starch and (iii) protein digestibility contribute greatly to the total variability among the sorghum varieties studied. In view of this finding, it was of interest to compare patterns of *in vitro* pepsin digestion using waxy and non-waxy lines that represent the widest variation for these traits. It was expected that these analyses might yield clues about the control of sorghum digestibility.

A composite of NuPAGE gels of four waxy and four non-waxy sorghum lines reveals patterns of storage proteins resistant to *in vitro* pepsin digestion over 3 h at 37 °C (Fig. 6). The choice of sorghum lines and their placement in the figure are an attempt to show the effects on protein digestion pattern of: (a) grain of low to high amylose content, (b) grain of low to high starch content, and (c) grain of low to high protein digestibility.

Variations in the protein patterns can best be demonstrated by dividing gels into regions of high molecular weight proteins [HMW] (>40 kDa) and low molecular weight proteins [LMW] (<30 kDa). The HMW region, which contains mostly glutelins and some oligomers of kafirin (Fig. 5), is considered to consist of elements that contribute to the protein matrix [31]. The LMW region consists mainly of α1-, α2-, γ- and β-kafirins (Fig. 5), the major components comprising the protein body.

The non-waxy pair, KS51 and 05-3771-4N with 22.5 and 32% amylose, respectively, had similar protein patterns in both high and low MW regions (Fig. 6A and B). The patterns for the waxy pair, BTx630 vs. BTxAGR1 with 5.7% vs. 11.8% amylose, respectively, were also found to be similar to each other (Fig. 6C and D, respectively). However, patterns in the HMW and LMW regions differed when compared to lines with different starch types, especially the LMW regions of waxy lines. The low-starch, non-waxy line, RTx430 (56% starch), has a notably different pattern in both regions relative to that of the high-starch, non-waxy line, Wheatland (85% starch) (Fig. 6E and F, respectively). The low-starch, waxy line, KS48 (47.9%), and the high-starch, non-waxy line 05-3771-1W (80.9%) had very similar protein patterns in both HMW and LMW regions, although digestion of various kafirins was faster in KS48
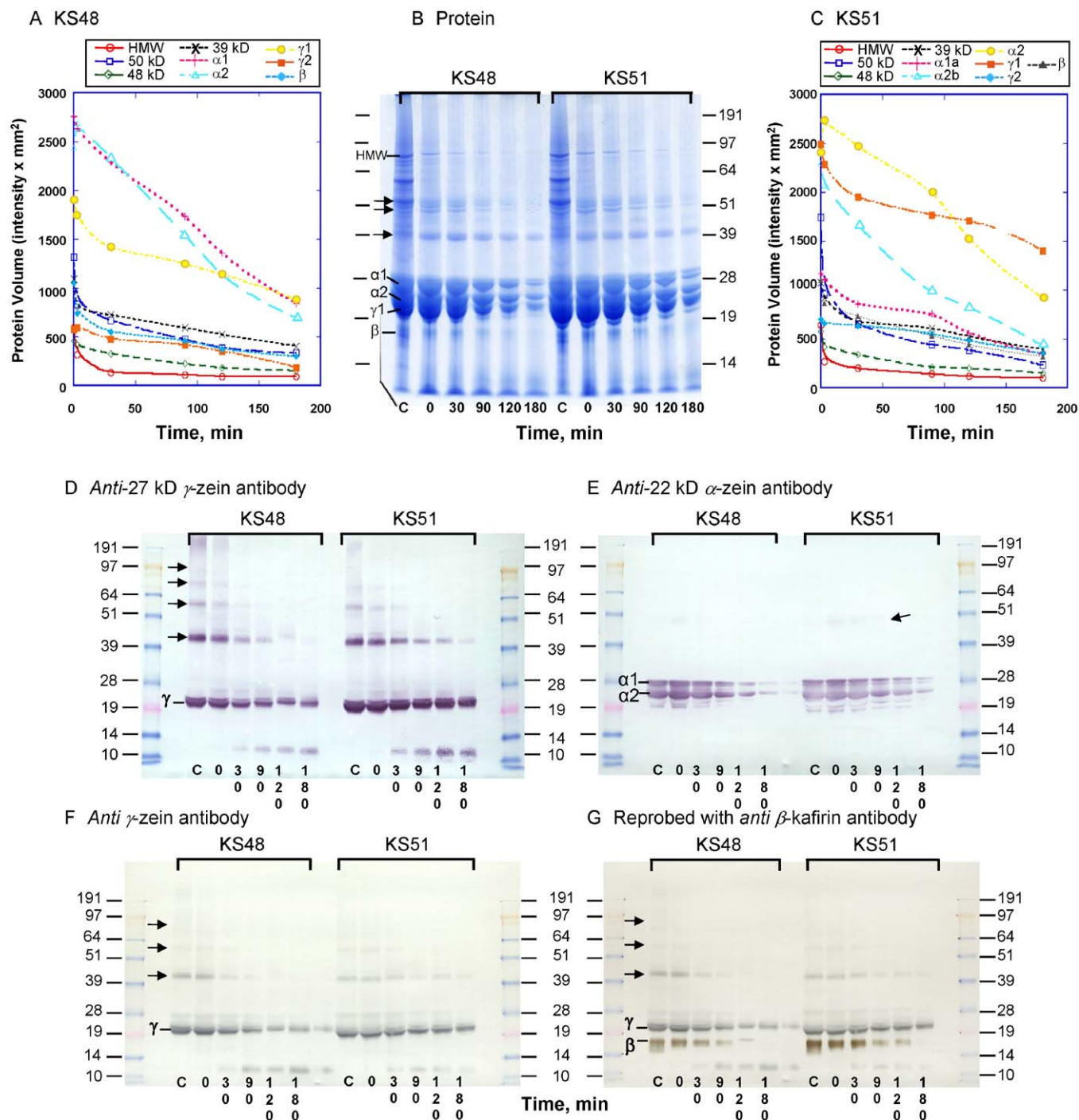
Fig. 5. Pepsin digestion patterns of monomeric and polymeric forms of α-, β- and γ-kafirins from a pair of sorghum lines visualized using western blots. Volume of protein-band quantification of (A) KS48 and (C) KS51, based on (B) NuPAGE gel of *in vitro* pepsin digestion of KS48 (waxy) and KS51 (non-waxy) sorghum lines. Western blots probed with (D) mono-specific anti 27 kDa γ-zein and (E) 22 kDa α-zein antibodies; (F) polyclonal anti γ-zein antibody and (G) blot of (F) re-probed with anti β-kafirin antibody. Molecular weight standards are from SeeBlue Plus2 Pre-stained standard (Invitrogen, Carlsbad, CA).

(Fig. 6G and H, respectively). The non-waxy line, Wheatland, with the lowest protein digestibility (34.6%), had strikingly different protein digestion patterns in both the HMW and LMW regions, relative to those of another non-waxy line, KS51, which had high protein digestibility (56.2%) (Fig. 6I and J, respectively). Like the non-waxy pair, the waxy line, BTx630 with low protein digestibility (38.4%), had strikingly different protein digestion patterns in both HMW and LMW regions, relative to the waxy KS48 line with high protein digestibility, 62.5% (Fig. 6K and L, respectively). The unusual protein digestion patterns of Wheatland and BTx630 with their low

protein digestibility (Table 2) may be a coincidental observation that requires further investigation.

Based solely on the analysis of these eight sorghum lines, apparent differences in the protein matrix, as well as differences in protein body composition, appear to relate to variations in their digestibility. These observations, however, fall short of establishing a cause and effect relationship, because effects of genetic diversity among these lines must also be considered. Research on additional sorghum lines using the methods applied in the present study is needed to confirm the present observations.
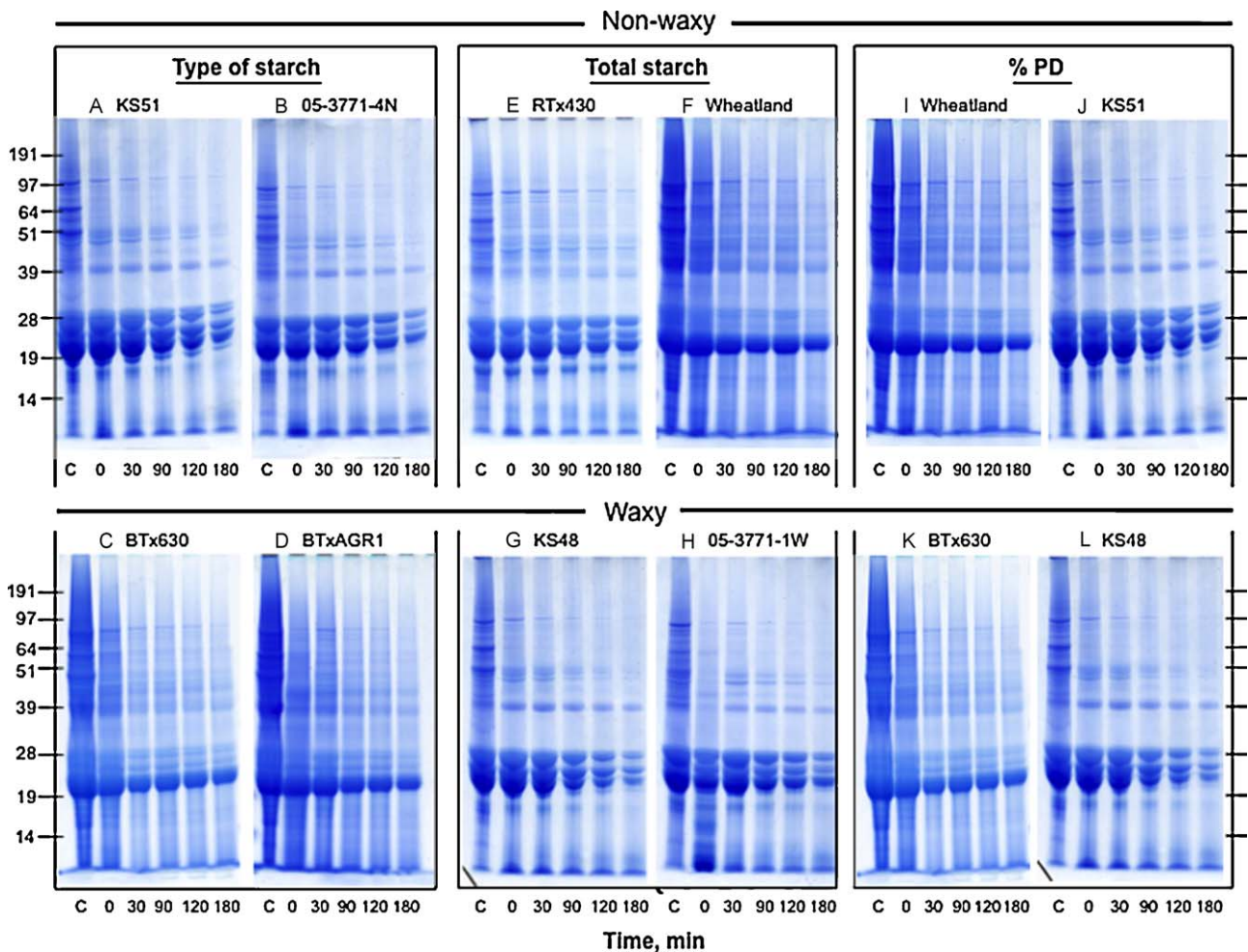
Fig. 6. Comparison of *in vitro* protein digestion patterns by pepsin of sorghum lines at opposing ends of the three categories identified by PCA: type of starch, total starch and % protein digestibility. Four non-waxy (KS51, 05-3771-4N, RTx430, and Wheatland; top panel) and four waxy (BTx630, BTxAGR1, KS48, and 05-3771-1W; bottom panel) sorghum lines are included.

The results of this study pertaining to the importance of the principal component analysis, together with the relative digestibility of different kafirins detected immunologically, suggest that future work on improving digestibility should focus on under- and over-expressing genes for specific storage proteins and enzymes of starch synthesis. These studies are expected to lead to more controlled differences in starch granule morphology and endosperm structure that because of their directed nature will be easier to analyze.

4. Conclusions

- Much of the variation in our sample population can be attributed to starch type (amylose vs. amylopectin), starch content and protein digestibility (12-h IVDMD and pepsin assays). Although each digestibility analysis examined in this study is purportedly optimal for a specific application, a combination of the four analyses identified offers clear approaches to identify targets for genetically improving the digestibility of sorghum grain.
- The molecular basis for the two known waxy alleles and their effects on GBSS activity have been established [54]; however, it appears that the type of starch has a differential effect on overall protein digestion patterns – an observation that needs to be better understood.
- Starch content affects digestion patterns of the protein matrix and makeup of the protein body to a greater extent in non-waxy than

waxy lines. Up-regulation of genes associated with starch accumulation is likely to identify targets for improving digestibility.
- Multiple forms of kafirins remain undigested by pepsin after 3 h. A γ-kafirin of M_r 20 kDa, the major undigested component, is a likely target for down-regulation to improve digestibility. This γ-kafirin is smaller than the α1- and α2-kafirins, M_r 28 and 25 kDa, respectively, but is larger than the readily digested 18 kDa β-kafirin.
- The %PD is influenced by the protein matrix, but more significantly by protein body packaging. Both the protein matrix and the distribution of α-, β- and γ-kafirins in the protein body vary among waxy and non-waxy sorghums in a way that affects digestibility. Understanding the complex interactions among components of the protein matrix would be enhanced by precise modification of genes for these proteins.
- P898012 is a unique testa-containing sorghum line with high digestibility. Further research is warranted on the presumed tannin(s) in its testa layer and their relationship to starch and protein digestibility.

References

[1] T. Walker, Sorghum grain for poultry and swine, ASA Technical Bulletin AN-20 (1999) 1–10.

[2] W.C. MacLean, G.L. de Romana, R.P. Placko, G.G. Graham, Protein quality and digestibility of sorghum in preschool children: balance studies and plasma free amino acids, Journal of Nutrition 111 (1981) 1928–1936.

[3] J.D. Axtell, A. Kirleis, M.M. Hassen, N. D'Croz-Mason, E.T. Mertz, L. Munck, Digestibility of sorghum proteins, Proceedings of the National Academy of Sciences of the United States of America 78 (1981) 1333–1335.

[4] B.R. Hamaker, A.W. Kirleis, E.T. Mertz, J.D. Axtell, Effect of cooking on the protein profiles and in vitro digestibility of sorghum and maize, Journal of Agricultural and Food Chemistry 34 (1986) 647–649.

[5] L.W. Rooney, R.L. Pflugfelder, Factors affecting starch digestibility with special emphasis on sorghum and corn, Journal of Animal Science 63 (1986) 1607–1623.

[6] S.F. Kotarski, R.D. Waniska, K.K. Thurn, Starch hydrolysis by the ruminal microflora, Journal of Nutrition 122 (1992) 178–190.

[7] M. Benmoussa, B. Suhendra, A. Aboubacar, B.R. Hamaker, Distinctive sorghum starch granule morphologies appear to improve raw starch digestibility, Starch/Stärke 58 (2006) 92–99.

[8] J.F. Pedersen, S.R. Bean, R.A. Graybosch, S.H. Park, M. Tilley, Characterization of waxy grain sorghum lines in relation to granule-bound starch synthase, Euphytica 144 (2005) 151–156.

[9] K. Denyer, P. Johnson, S. Zeeman, A.M. Smith, The control of amylose synthesis, Journal of Plant Physiology 158 (2001) 479–487.

[10] R.F. Tester, J. Karkalas, X. Qi, Starch structure and digestibility enzyme–substrate relationship, World's Poultry Science Journal 60 (2004) 186–195.

[11] J.F. Pedersen, R.A. Graybosch, D.L. Funnell, Occurrence of the waxy alleles wx^a and wx^b in waxy sorghum plant introductions and their effect on starch thermal properties, Crop Science 47 (2007) 1927–1933.

[12] L.W. Rooney, F.R. Miller, Variation in the structure and kernel characteristics of sorghum, in: International Symposium on Sorghum Grain Quality, ICRISAT Center, Patancheru, India (1982), pp. 143–162.

[13] A. Chandrashekar, H. Mazhar, The biochemical basis and implications of grain strength in sorghum and maize, Journal of Cereal Science 30 (1999) 193–207.

[14] K.G. Duodu, A. Nunes, I. Delgadillo, M.L. Parker, E.N.C. Mills, P.S. Belton, J.R.N. Taylor, Effect of grain structure and cooking on sorghum and maize in vitro protein digestibility, Journal of Cereal Science 35 (2002) 161–174.

[15] B.R. Hamaker, A.A. Mohamed, J.E. Habben, C.-P. Huang, B.A. Larkins, An efficient procedure for extracting maize and sorghum kernel proteins reveals higher prolamin contents than the conventional method, Cereal Chemistry 72 (1995) 583–588.

[16] M.P. Oria, B.R. Hamaker, J.D. Axtell, C.-P. Huang, A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm protein bodies, Proceedings of the National Academy of Sciences of the United States of America 97 (2000) 5065–5070.

[17] J.M. Shull, J.J. Watterson, A.W. Kirleis, Purification and immunocytochemical localization of kafirins in Sorghum bicolor (L. Moench) endosperm, Protoplasma 171 (1992) 64–74.

[18] A. Aboubacar, J.D. Axtell, C.-P. Huang, B.R. Hamaker, A rapid protein digestibility assay for identifying highly digestible sorghum lines, Cereal Chemistry 78 (2001) 160–165.

[19] B.R. Hamaker, A.W. Kirleis, L.G. Butler, J.D. Axtell, E.T. Mertz, Improving the in vitro protein digestibility of sorghum with reducing agents, Proceedings of the National Academy of Sciences of the United States of America 84 (1987) 626–628.

[20] M.P. Oria, B.R. Hamaker, J.M. Shull, Resistance of sorghum α-, β- and γ-kafirins to pepsin digestion, Journal of Agricultural and Food Chemistry 43 (1995) 2148–2152.

[21] A.E.O. Elkhalifa, A. Chandrashekar, B.E. Mohamed, A.H. El Tinay, Effect of reducing agents on the in vitro protein and starch digestibilities of cooked sorghum, Food Chemistry 66 (1999) 323–326.

[22] J.M. Shull, A. Chandrashekar, A.W. Kirleis, G. Ejeta, Development of sorghum [Sorghum bicolor (L.) Moench] endosperm in varieties of varying hardness, Food Structure 9 (1990) 253–267.

[23] A. Chandrashekar, A.W. Kirleis, Influence of protein on starch gelatinization in sorghum, Cereal Chemistry 65 (1988) 457–462.

[24] G. Zhang, B.R. Hamaker, Low α-amylase starch digestibility of cooked sorghum flours and the effect of protein, Cereal Chemistry 75 (1998) 710–713.

[25] K.G. Duodu, J.R.N. Taylor, P.S. Belton, B.R. Hamaker, Factors affecting sorghum protein digestibility, Journal of Cereal Science 38 (2003) 117–131.

[26] L.I. Ezeogu, K.G. Duodu, J.R.N. Taylor, Effects of endosperm texture and cooking conditions on the in vitro starch digestibility of sorghum and maize flours, Journal of Cereal Science 42 (2005) 33–44.

[27] J. Wong, T. Lau, N. Cai, J. Singh, J.F. Pedersen, J. Wilson, W.H. Vensel, W.J. Hurkman, P.G. Lemaux, B.B. Buchanan, Digestibility of protein and starch from sorghum (Sorghum bicolor) is linked to biochemical and structural features of grain endosperm, Journal of Cereal Science 49 (2009) 73–82.

[28] N.D. Luchini, G.A. Broderick, D.K. Combs, Characterization of the proteolytic activity of commercial proteases and strained ruminal fluid, Journal of Animal Science 74 (1996) 685–692.

[29] J.F. Pedersen, T. Milton, R.A. Mass, A twelve-hour in vitro procedure for sorghum grain feed quality assessment, Crop Science 40 (2000) 204–208.

[30] E.T. Mertz, M.M. Hassen, C. Cairn-Whittern, A.W. Kirleis, L. Tu, J.D. Axtell, Pepsin digestibility of proteins in sorghum and other major cereals, Proceedings of the National Academy of Sciences of the United States of America 81 (1984) 1–2.

[31] B.R. Hamaker, B.A. Bugusu, Overview: sorghum proteins and food quality, in: P.S. Belton, J.R.N. Taylor (Eds.), Workshop on the Proteins of Sorghum and Millets: Enhancing Nutritional and Functional Properties for Africa [CD], Pretoria, South Africa, 2003.

[32] L.I. Ezeogu, K.G. Duodu, M.N. Emmanbux, J.R.N. Taylor, Influence of cooking conditions on the protein matrix of sorghum and maize flours, Cereal Chemistry 85 (2008) 397–402.

[33] T.-J. Fu, U.R. Abbott, C. Hatzos, Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid – a comparative study, Journal of Agricultural and Food Chemistry 50 (2002) 7154–7160.

[34] A.V. Berdutina, A.D. Neklyudov, A.I. Ivankin, B.S. Karpo, S.I. Mitaleva, Comparison of proteolytic activities of the enzyme complex from mammalian pancreas and pancreatin, Applied Biochemistry and Microbiology 36 (2000) 363–367.

[35] P. Bernfeld, Amylases α and β, Academic Press, New York, 1955.

[36] J. Mosse, 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 (1990) 18–24.

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

[38] Anonymous, Simulated Gastric Fluid and Simulated Intestinal Fluid, The Unites States Pharmacopeial Convention, Inc., Rockville, MD, 1995.

[39] S.R. Bean, Evaluation of novel pre-cast gradient gels for the separation of sorghum proteins, Cereal Chemistry 80 (2003) 500–504.

[40] S. Krajewski, J.M. Zapata, J.C. CReed, Detection of multiple antigens on western blots, Analytical Biochemistry 236 (1996) 221–228.

[41] T. Nakamura, P. Vrinten, K. Hayakawa, J. Ikeda, Characterization of a granule-bound starch synthase isoform found in pericarp of wheat, Plant Physiology 118 (1998) 451–459.

[42] J.F. Pedersen, S.R. Bean, D.L. Funnell, R.A. Graybosch, Rapid iodine staining techniques for identifying the waxy phenotype in sorghum grain and waxy genotype in sorghum pollen, Crop Science 44 (2004) 764–767.

[43] L. Dykes, L.W. Rooney, Sorghum and millet phenols and antioxidants, Journal of Cereal Science 44 (2006) 236–251.

[44] M.P. Oria, B.R. Hamaker, J.M. Shull, 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 (1995) 85–93.

[45] P.R. Shewry, N.G. Halford, The prolamin storage proteins of sorghum and millets, in: P.S. Belton, J.R.N. Taylor (Eds.), AFRIPRO, Pretoria, South Africa, 2003.

[46] P.S. Belton, I. Delgadillo, N.G. Halford, P.R. Shewry, Kafirin structure and functionality, Journal of Cereal Science 44 (2006) 272–286.

[47] C. Hicks, S.R. Bean, G.L. Lookhart, J.F. Pedersen, K.D. Kofoid, M.R. Tuinstra, Genetic analysis of kafirins and their phenotypic correlations with feed quality traits, in vitro digestibility, and seed weight in grain sorghum, Cereal Chemistry 78 (2001) 412–416.

[48] J.M. Shull, J.J. Watterson, A.W. Kirleis, 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 (1991) 83–87.

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

[50] J.J. Watterson, J.M. Shull, A.W. Kirleis, Quantitation of α-, β- and γ-kafirins in vitreous and opaque endosperm of Sorghum bicolor, Cereal Chemistry 70 (1993) 452–457.

[51] L. Izquierdo, I.D. Godwin, Molecular characterization of novel methionine-rich delta-kafirin seed storage protein gene in sorghum (Sorghum bicolor L.), Cereal Chemistry 82 (2005) 706–710.

[52] Y.-M. Woo, D.W.-N. Hu, B.A. Larkins, H.R. Jung, Genomics analysis of genes expressed in maize endosperm identifies novel seed proteins and clarifies patterns of zein gene expression, The Plant Cell 13 (2001) 2297–2317.

[53] A.J. Casady, 10 Sorghum parental lines, Crop Science 12 (1972) 722.

[54] S.E. Sattler, J. Singh, E.J. Haas, L. Guo, G. Sarath, J.F. Pedersen, Two distinct waxy alleles impact the granule-bound starch synthase in sorghum, Molecular Breeding 24 (2009) 349–359.

[55] D.T. Rosenow, J. Moore, M.A. Hussey, Rosenow Sorghum Parental Line Release, TAES/TAMU, Lubbock/College Station, TX, 2002.

[56] MP-510, An Evaluation of Parents of Grain Sorghum Hybrids, Texas A& M College, Texas Agricultural Experiment Station/USDA, College Station, TX, 1961.

[57] R.E. Atkins, Registration of 12 sorghum parental lines, Crop Science 23 (1983) 1229.
[58] F.R. Miller, Registration of RTx430 sorghum parental line, Crop Science 24 (1984) 1224.
[59] H.B. Brown, J.A. Clark, E.F. Gaines, H.K. Hayes, W.J. Morse, J.H. Parker, T.R. Stanton, G.H. Stringfield, M.A. McCall, Varietal standardization and registration, Journal of the American Society of Agronomy 28 (1936) 1027.
[60] F.R. Miller, Registration of RTx435 sorghum parental line, Crop Science 26 (1986) 215–216.
[61] W.M. Ross, H.J. Gorz, F.A. Haskins, O.J. Webster, Registration of ten sorghum parental lines (Reg. No. PL49 to PL5), Crop Science 20 (1980) 834.
[62] F.R. Miller, C. Domanski, L.M. Giorda, Registration of A/BTxARG-1 sorghum, Crop Science 32 (1992) 1517.
[63] F.R. Miller, K.L. Prihoda, Registration of 16 sorghum germplasm lines: Tx2892 through Tx2906 and Tx2908, Crop Science 36 (1996) 476–477.
[64] W.A. Russell, Registration of B70 and B73 parental lines of maize (Reg. Nos. PL16 and PL17), Crop Science 12 (1972) 721.