



Digestibility of protein and starch from sorghum (*Sorghum bicolor*) is linked to biochemical and structural features of grain endosperm

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ABSTRACT

Although a principal source of energy and protein for millions of the world's poorest people, the nutritional value of sorghum (*Sorghum bicolor* L. Moench) is diminished because of low digestibility of grain protein and starch. To address this problem, we analyzed the properties of two sorghum lines that have a common pedigree but differ in digestibility. Consistent with results based on a ruminal fluid assay, the protein and starch of one line (KS48) was more thoroughly digested than that of the other (KS51) using *in vitro* assays based on pepsin and α -amylase. The indigestibility of KS51 relative to KS48 was shown to be due to (i) a greater abundance of disulfide-bonded proteins; (ii) presence in KS51 of non-waxy starch and the accompanying granule-bound starch synthase; and (iii) the differing nature of the protein matrix and its interaction with starch. The current findings suggest that each of these factors should be considered in efforts to enhance the nutritional value of sorghum grain.

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1. Introduction

Grain sorghum (*Sorghum bicolor* L. Moench) ranks fifth in worldwide production among cereal crops, after wheat, rice, corn and barley. The U.S. is the number two producer and the number one exporter of sorghum, primarily to Mexico for use as animal feed (U.S. Grains Council, 2006). Popularity of sorghum is due in part to its ability to produce reasonable yields in warmer, drier regions. Because subsistence farmers in Africa and Asia cultivate sorghum widely as a staple food for home consumption, the crop is

a principal source of energy and protein for millions of the world's poorest (Klopfenstein and Hoseney, 1995).

Seed proteins of sorghum are less digestible than those of other cereals and digestibility is exacerbated by wet cooking the meal or flour, which results in significant nutritional losses (Hamaker et al., 1986; MacLean et al., 1981; Mertz et al., 1984). Numerous factors contribute to the digestibility problem [for review, see (Duodu et al., 2003)]. Exogenous aspects include the interaction of protein with non-protein components, such as polyphenols, starch, non-starch polysaccharides, phytates and lipids. Endogenous factors arise from the nature of the proteins themselves and their organization within the grain (Belton et al., 2006; Duodu et al., 2003; Ezeogu et al., 2005, 2008; Hamaker and Bugusu, 2003). As in other cereals, the low amount of protein relative to starch, *i.e.*, approximately 10% protein, vs. 70–80% starch, based on grain dry weight (Rooney and Pflugfelder, 1986), affects the functional properties of starch, such as gelatinization and digestion rate, to a greater extent in sorghum than in other cereals (Chandrashekar and Kirleis, 1988; Duodu et al., 2002; Ezeogu et al., 2005, 2008).

The proteins of the sorghum grain are classically divided, based on solubility in different solvents (Jambunathan et al., 1975; Landry and Moureaux, 1970): albumins (water-soluble), globulins (salt-soluble), kafirins (prolamins, aqueous alcohol-soluble), cross-linked kafirins (aqueous alcohol + reducing agent-soluble), cross-linked glutelins (detergent + reducing agent + alkaline pH-soluble) and unextracted

Abbreviations: DTT, dithiothreitol; GBSS, granule-bound starch synthase; IVDMD, *in vitro* dry matter disappearance; ME, 2-mercaptoethanol; M_r , relative molecular mass; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino) propane sulfonic acid.

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structural protein residue. A newer and more simplified classification scheme for sorghum proteins has been proposed that divides them into two groups, kafirins and non-kafirins. This scheme is based on the homogeneous nature and varied origin of the kafirin storage prolamins relative to the heterogeneous nature of the non-kafirin proteins (i.e., albumins, globulins and glutelins) that are involved in cellular functions (Hamaker and Bugusu, 2003; Hamaker et al., 1995). Despite a high degree of sequence homology to maize zeins, sorghum storage proteins contain a higher proportion of cross-linked fractions and are more hydrophobic, explaining their greater propensity to form intermolecular disulfide-cross linkages and possibly additional protein aggregates that could facilitate the formation of more covalent bonds compared to maize zeins (Belton et al., 2006; Hamaker and Bugusu, 2003).

Kafirins, comprising 70–80% of the protein in whole grain sorghum flour (Hamaker et al., 1995), are synthesized and translocated into the lumen of the endoplasmic reticulum where they form protein bodies (Taylor et al., 1985). They are subclassified as: α - (23 and 25 kDa), β - (20 kDa) and γ - (28 kDa) types based on molecular weight, extractability, structure and cross-reactivity with sera against analogous maize zeins (Belton et al., 2006; Mazhar et al., 1993; Shull et al., 1991). Comprising ~80% of total kafirins, the α -type is considered the principal storage protein, followed by the γ - (~15%) and β - (~5%) members. Recently, a DNA-derived sequence for a δ -kafirin was shown to have high homology with the M_r 10,000 δ -zein, except for absence of part of the methionine-rich region (Belton et al., 2006; Izquierdo and Godwin, 2005). α -Kafirins have low levels of cysteine relative to the β - and γ -types (5 and 7 mol%, respectively), whereas δ -kafirins are rich in methionine (16–18 mol%) but lack cysteine. Electron microscopy of internal protein body structure reveals that γ -kafirins, and to a lesser extent β -kafirins, encapsulate the more digestible α -kafirins in a disulfide-bond polymer network (Oria et al., 2000; Shull et al., 1992), thereby impeding exposure to proteases.

In the corneous endosperm, non-kafirins (albumins, globulins, glutelins) form around protein bodies, effectively “gluing” the bodies into a matrix surrounding the starch granules (Hamaker and Bugusu, 2003; Shull et al., 1990; Taylor et al., 1984). This protein matrix appears to act as a barrier to starch gelatinization and digestibility (Chandrashekar and Kirleis, 1988; Duodu et al., 2002; Ezeogu et al., 2005, 2008) due to cross-linking between γ - and β -kafirins and matrix proteins (Duodu et al., 2001; Hamaker and Bugusu, 2003; Oria et al., 1995a). Cooking reduces digestibility by effecting a conformational change in proteins that could facilitate formation of disulfide-linked polymers (Axtell et al., 1981; Duodu et al., 2002, 2003; Hamaker et al., 1987; Oria et al., 1995b). The negative impact of cooking on protein digestibility was mitigated by addition of 2-mercaptoethanol (ME) or other reducing agents (Elkhalifa et al., 1999; Hamaker et al., 1987). Sorghum grains rich in kafirin-containing protein bodies also have a lower capacity for starch gelatinization (Chandrashekar and Kirleis, 1988; Ezeogu et al., 2005, 2008)—an observation consistent with the finding that adding ME during cooking to cleave disulfide bonds within the protein matrix increased the degree of starch gelatinization and digestion (Elkhalifa et al., 1999; Ezeogu et al., 2005, 2008; Zhang and Hamaker, 1998).

The protein barrier surrounding the starch granule may also reduce the hydrolysis of native and processed starch by amylolytic enzymes (Rooney and Pflugfelder, 1986). Thus, the addition of pronase to hydrolyze the protein matrix significantly enhanced *in vitro* rates of starch hydrolysis by increasing surface area and enabling starch to interact with α -amylase and amyloglucosidase (Rooney and Pflugfelder, 1986). Similar effects were observed when sorghum flour was either treated with pepsin before cooking or cooked in the presence of dithiothreitol (DTT) or other reductants (Zhang and Hamaker, 1998). Abundance of starch granules may also

decrease proteolysis by limiting accessibility of proteolytic enzymes, especially when gelatinized during cooking (Duodu et al., 2002). Uniqueness of the protein matrix and its interaction with starch that affect the rate of starch digestion are key differences between the feed quality of sorghum and corn (Rooney and Miller, 1982; Rooney and Pflugfelder, 1986). In addition, endosperm texture and cooking conditions have been shown to have a significant effect on *in vitro* digestibility of starch and protein in these two cereals (Duodu et al., 2002, 2003; Ezeogu et al., 2005, 2008). Sorghum displays significant variation in rates of starch disappearance (Wester et al., 1992). Based on a 12-h incubation in the *in vitro* dry matter disappearance (IVDMD) assay, loss of dry matter in sorghum correlated closely with rates of starch, but not necessarily protein digestion (Pedersen et al., 2000).

Collectively, these findings suggest that properties of starch and protein in sorghum could affect their mutual digestibility. The majority of published references deal with digestibility of the proteins and their impact on starch, but evidence for the reverse is scant (Duodu et al., 2002). To pursue this issue and assess the importance of relevant factors in a single study, we investigated how protein and starch influence the breakdown of one another by comparing two sorghum lines having a common pedigree but differing in digestibility.

2. Materials and methods

2.1. Grain and preparation of materials

The sorghum lines KS48 and KS51 were selected for these experiments because of previously determined differences in digestibility but the identical pedigree, Texioca-63 \times Short Kaura (Casady, 1972). Both have a clear pericarp and yellow endosperm. Field-grown seeds were produced at the University of Nebraska Agricultural Research Development Center, Ithaca, NE; additional seeds were generated from greenhouse-grown plants at the University of California, Berkeley. Seed size and hardness of KS48 and KS51 were assessed using a Perten SKCS 4100 Single Kernel Characterization System instrument. The starch content of KS48 and KS51 was 64.7% and 67.8%, respectively, on a dry weight basis. For 12-h IVDMD analysis, mature dry seeds were ground in a Wiley mill to pass a 20-mesh screen. For all other analyses, seeds were ground in a Wiley mill to pass a 40-mesh screen. Ground meal was stored at 25 °C until used in digestion assays. Pepsin (porcine stomach mucosa, P-7000) and three types of α -amylases – bacterial (A-3403, Type XII-A), porcine pancreas (A-3176, Type VI-B) and human saliva (A-1031, Type XIII-A) – were used in digestibility assays (Sigma, St Louis, MO).

2.2. Microscopy studies

For microscopic observations, seeds were sectioned with a razor blade and stained with iodine (0.3 g I₂, 1.0 g KI in 100 ml water). Sectioned seeds were used for electron microscopic analyses, using a Hitachi TM 1000 environmental scanning electron microscope (Hitachi High Technologies America, Inc., Pleasanton, CA). Prior to analysis, sections were coated with gold-palladium to 1 mm using a Tousimis sputter machine (Rockville, MD).

2.3. Twelve-hour IVDMD of sorghum meal

Sorghum meal was digested for 12 h in rumen fluid inoculum obtained from a ruminally fistulated steer using previously described methods (Pedersen et al., 2000).

2.4. Sequential protein extraction

Sorghum meal (2 g) was sequentially extracted with the six solvents listed below in 20 ml lots at 25 °C and centrifuged (18,900g, 10 min) at 4 °C after each step [modified from (Hamaker et al., 1986)]. Initially, meal was extracted with 0.5 M NaCl by shaking for 60 min. Resulting NaCl-soluble albumins and globulins (fraction 1) were retained, and the pellet sequentially extracted yielding the following supernates: double distilled H₂O (ddH₂O) for 20 min (water-wash fraction, fraction 2); 60% 2-propanol (v/v) for 4 h (kafirins, fraction 3); 0.1 M borate buffer, pH 10.8, for 4 h (glutelin-like protein, fraction 4); 60% 2-propanol with 1% dithiothreitol (DTT) for 4 h (cross-linked kafirins, fraction 5); and 0.1 M borate buffer, pH 10.8, containing 1% DTT and 1% sodium dodecyl sulfate (SDS) for 18 h at 4 °C (cross-linked glutelins, fraction 6). Protein fractions were stored at –20 °C.

2.5. Disulfide protein content of selected fractions

Equal amounts of protein in fractions extracted without reducing agent were reduced with 1 mM DTT; protein sulfhydryl groups were labeled with monobromobimane (mBBR) and analyzed using SDS-PAGE (Wong et al., 2004). Relative intensity of fluorescence of labeled proteins was equated to relative amount of protein disulfides.

2.6. Rapid *in vitro* pepsin digestion

Time course of *in vitro* pepsin digestion of sorghum meal and processing of the resulting undigested residue were as described (Aboubacar et al., 2001; Nunes et al., 2004).

2.7. Extraction of protein undigested by pepsin

Protein in pellets, not digested by pepsin, was extracted with 0.0125 M borate buffer, pH 10 containing 1% SDS and 2% 2-ME as described (Aboubacar et al., 2001). The extraction was modified further to include extraction of a granule-bound starch synthase I (GBSSI) from the borate–SDS–ME extracted residue (see Section 2.8).

2.8. Extraction of granule-bound starch synthase

To determine if unextracted protein remained in residues after extraction with borate–SDS–ME (0.0125 M borate, 1% SDS, 2% ME, pH 10), the washed pellets (in 0.5 ml of the same buffer) were mixed with 1 ml borate–SDS–ME buffer and extracted for 5–10 min in a boiling water bath and cooled in tap water. Gelatinized starch was pelleted by centrifugation (20,800g, 10 min) and the clear supernate was saved for SDS-PAGE analysis.

2.9. SDS-PAGE

A Criterion Pre-Cast Gel System using Tris–HCl gels (Bio-Rad, Hercules, CA) was used for SDS-PAGE analysis (Wong et al., 2004). Equal amounts of protein were used for protein distribution analyses and estimation of sulfhydryl content. For protein digestion analysis, equal volumes (~5 µl) with a specified amount of extracted protein were used.

2.10. SDS-PAGE of kafirins and glutelins undigested by pepsin

NuPAGE Novex Bis–Tris gels (Invitrogen, Carlsbad, CA), with a neutral pH to minimize protein modifications, were used to obtain optimal resolution of small- to medium-sized proteins. Aliquots (~5 µl) of specific amounts of extracted protein were mixed with 1/4 volume of 4× NuPAGE sample buffer plus 0.4% ME,

boiled 5 min, centrifuged and run in 12% Bis–Tris gels with MOPS buffer for 1 h 20 min at 150 V. Fluorescent imaging and protein staining were as described (Section 2.11).

2.11. Quantification of fluorescent proteins

To determine redox status, fluorescent (unsaturated and saturated) images of reduced proteins were captured from gels on a Gel Doc 1000 imager using Quantity One software (Bio-Rad, Hercules, CA) with 365 nm UV light either immediately or after a 15 min water-wash; images were saved in TIFF format. Gels were stained for protein with colloidal Coomassie Blue G-250 overnight at 25 °C, destained in ddH₂O and scanned with a UMAX PowerLook 1100 scanner using UMAX MagicScan version 4.4 within Adobe Photoshop, v.6 (Adobe Systems, San Jose, CA). Amount of protein, expressed as volume (intensity × area) of fluorescent bands and/or undigested kafirin bands, was quantified with Quantity One software. Higher protein band volume indicates more protein in the gel, and thus lower digestibility (Aboubacar et al., 2001).

2.12. 2-D gel electrophoresis

Protein contained in equal volumes of extracts was precipitated by addition of 5 vol of cold (–20 °C) acetone and incubation at –20 °C overnight. Proteins were recovered by centrifugation and separated by 2-D gel electrophoresis (Wong et al., 2004).

2.13. Western blot analysis

After electrophoresis, proteins were transferred to nitrocellulose membranes at 4 °C for 75 min at 50 V constant voltage (Wong et al., 2004) and cross-reactivity analyzed with waxy protein antibody (Terada et al., 2000).

2.14. *In vitro* starch digestion

Digestibility of starch in uncooked sorghum meal was measured using a modified hydrolysis method (Zhang and Hamaker, 1998) with bacterial α -amylase (A-3403, Type XII-A, Sigma Chemical Co., St. Louis, MO).

2.15. Sequential addition of α -amylase/pepsin or pepsin/ α -amylase

2.15.1. α -Amylase followed by pepsin

Meal (50 mg) was treated with 50 µg α -amylase (human saliva form, Sigma A-1031, Type XIII-A) in 0.3 ml 1 mM Na glycerophosphate–HCl buffer (25 mM NaCl, 5 mM CaCl₂, 0.02% w/v azide), pH 6.9, at 37 °C for 1 h with shaking. Pepsin solution (1 ml at 20 mg/ml, pH 2.0) was added, the mixture incubated for 2 h at 37 °C and neutralized with 0.1 ml 2 N NaOH. Supernate containing α -amylase- and pepsin-digested material was collected by centrifugation (20,800g, 10 min) for reducing sugar determination. Pellet was washed with 1 ml 0.1 M KH₂PO₄ buffer, pH 7.0 and then 1 ml ddH₂O and extracted for remaining kafirins and GBSSI as above. Controls were: (A) α -amylase alone, (B) pepsin alone and (C) buffer alone.

2.15.2. Pepsin followed by α -amylase

Meal (50 mg) was incubated with pepsin (1 ml at 20 mg/ml, pH 2.0) for 2 h at 37 °C with shaking and then neutralized (0.1 ml 2 N NaOH to pH 7.0). α -Amylase (50 µg) in 0.3 ml Na glycerophosphate buffer as above, was added and the mixture incubated for 1 h at 37 °C. Reaction was stopped by acidification and the supernate containing pepsin- and α -amylase-digestible materials was saved for reducing sugar determination. Pellets were washed 2× with 1 ml ddH₂O and extracted for kafirins and GBSSI (Sections 2.7 and 2.8). Controls were: (A) pepsin alone, (B) α -amylase alone and (C) buffer alone.

2.16. Effect of DTT reduction on digestion of cross-linked kafirins and glutelins by pepsin

Meal (50 mg) was incubated ± 5 mM DTT in 0.5 ml of 0.1 M KH_2PO_4 , pH 7.0, for 1 h at 37 °C and supernate removed by centrifugation (20,800g, 10 min). The DTT-treated pellet was washed with 1 ml (A) ddH₂O and (B) 0.1 M KH_2PO_4 buffer, pH 2.0. The washed residue was incubated with pepsin (1 ml at 20 mg/ml in pH 2.0 KH_2PO_4 buffer) at 37 °C for 2 h with shaking and digestion was stopped with 0.1 ml 2 N NaOH. Pepsin-treated residue was extracted for remaining kafirins and glutelins and analyzed by NuPAGE as above.

2.17. Granule-bound starch synthase (GBSSI) identification

Coomassie blue-stained spots were excised from 2-D gels and placed in a 96-well reaction tray. After proteins were destained, reduced, alkylated and cleaved with trypsin by a robotic sample handler, they were analyzed by tandem mass spectrometry and identified as described (Balmer et al., 2006).

2.18. Other analytical procedures

Protein was estimated in buffer-soluble extracts by the dye-binding method and those in the presence of SDS and ME or DTT by the Non-Interfering Protein Assay (Geno Technology, Inc., St. Louis, MO) as described (Wong et al., 2004). The ratio of amylose and amylopectin was determined using a Megazyme™ Amylose/Amylopectin Assay Kit (Megazyme, Bray, Ireland).

3. Results and discussion

The 12-h IVDMD assay estimates the *in vitro* rate of starch digestion, which is highly correlated to feed efficiency or gain/feed ratio ($r=0.94$) (Stock et al., 1987). Because it mimics biochemical conditions in cattle, use of ruminal fluid containing hydrolytic enzymes produced by the ruminant and its microbial flora is relevant to studies of this type. The 12-h IVDMD applied to the two sorghum lines, KS48 and KS51, gave digestibility values of 33.0% and 22.1%, respectively. The results revealed a difference in dry matter disappearance and, hence, in the rates of digestion of starch as well as protein. The difference revealed by IVDMD, plus the common pedigree of these two lines, led to their choice for further study of digestibility. In the present work, grain from the two lines are subjected to *in vitro* pepsin and α -amylase assays that are generally considered to reflect activities of the human stomach and small intestine, respectively (Astwood et al., 1996; Zhang and Hamaker, 1998).

3.1. Protein distribution of KS48 and KS51

The distribution of protein in fractions extracted with the indicated solvents (Section 2.4) suggested that, in general, the two sorghum lines yielded approximately the same amount of total extractable protein (Table 1). However, differences in distribution of protein between the two lines suggested that the more digestible KS48 contains more protein than the less digestible KS51 in the first five fractions extracted—ddH₂O wash, albumin and globulin (salt-soluble), kafirin (60% 2-propanol), glutelin-like protein (borate soluble), and cross-linked kafirin (2-propanol + DTT). By contrast, the least soluble (last-extracted) fraction, cross-linked glutelin (borate + DTT + SDS), represented a considerably greater fraction in KS51 than in KS48. To determine whether these differences in protein distribution affect digestibility, we examined the composition and redox status of protein in the six fractions (Fig. 1).

Disulfide bonds between cysteine residues, a common feature of most cereal seed proteins, have long been known to stabilize various molecular structures (Wall, 1971). Many proteins with disulfide bonds resist digestion by proteases (Astwood et al., 1996; Opstvedt et al., 1984; del Val et al., 1999). Since a large percentage of sorghum kafirin storage proteins exist in polymeric forms linked by disulfide bonds in their native state (Duodu et al., 2002, 2003; El Nour et al., 1998; Hamaker et al., 1987; Oria et al., 1995b), differences in content of fractions rich in insoluble disulfide proteins, *i.e.*, 2-propanol + DTT (cross-linked kafirin) and borate + DTT + SDS (cross-linked glutelin) could contribute to protein digestibility differences. Accordingly, although KS48 had more 2-propanol + DTT extractable proteins (cross-linked kafirins), the content of borate + DTT + SDS-extractable glutelins was appreciably greater in KS51 than in KS48—a reflection of the relative abundance of intermolecular S–S groups in this fraction (Table 1). The latter could be a factor in the lower digestibility of KS51.

3.2. Estimation of disulfide protein status

Protein disulfide content was estimated by determining the sulfhydryl groups resulting from DTT reduction, using a thiol-specific fluorescent probe, mBBr (Wong et al., 2004). Four fractions (nos. 1–4 in Table 1), extracted without reducing agent, were monitored for DTT-induced sulfhydryl changes. KS51 showed more intense fluorescence relative to KS48 in fraction 1 salt (albumin and globulin) and fraction 4 borate (glutelin-like) (Fig. 1B). The more pronounced increase in proteins with disulfide bonds in fraction 6 (borate + DTT + SDS) could also contribute to the lower digestibility of KS51, as could the markedly different distribution of proteins in that fraction (Fig. 1A). For reasons not yet understood, the fluorescence intensity of the kafirin (propanol) fraction showed larger relative differences between the two lines in untreated vs. DTT-treated samples (Fig. 1B, lanes 9 vs. 11; 10 vs. 12). In summary,

Table 1
Protein distribution of two sorghum lines with a common pedigree differing in digestibility

| Fraction | Extraction solvent | KS48 ^a | | KS51 ^a | | KS48–KS51 Δ, % |
|----------|--|----------------------------|---------------------------|----------------------------|---------------------------|-------------------|
| | | Total mg protein extracted | % Total protein extracted | Total mg protein extracted | % Total protein extracted | |
| 1 | NaCl, 0.5 M | 28.3 ± 0.5 | 19.0 | 27.6 ± 0.4 | 18.8 | 0.2 |
| 2 | H ₂ O-wash | 0.4 ± 1.0 | 0.3 | 0.4 ± 1.4 | 0.3 | 0.0 |
| 3 | 2-Propanol, 60% | 17.3 ± 1.0 | 11.6 | 12.4 ± 1.3 | 8.5 | 3.1 |
| 4 | Borate, 0.1 M pH 10.8 | 5.7 ± 0.7 | 3.8 | 4.4 ± 1.2 | 3.0 | 0.8 |
| 5 | 2-Propanol, 60% + 1% DTT | 30.9 ± 2.8 | 20.8 | 27.8 ± 4.8 | 19.0 | 1.8 |
| 6 | Borate, 0.1 M pH 10.8 + 1% DTT + 1% SDS | 66.3 ± 3.1 | 44.5 | 73.8 ± 4.3 | 50.4 | –5.9 |
| Total | | 148.9 ± 1.5 | 100 | 146.4 ± 2.2 | 100 | – |

^a Mean of 5 experiments.

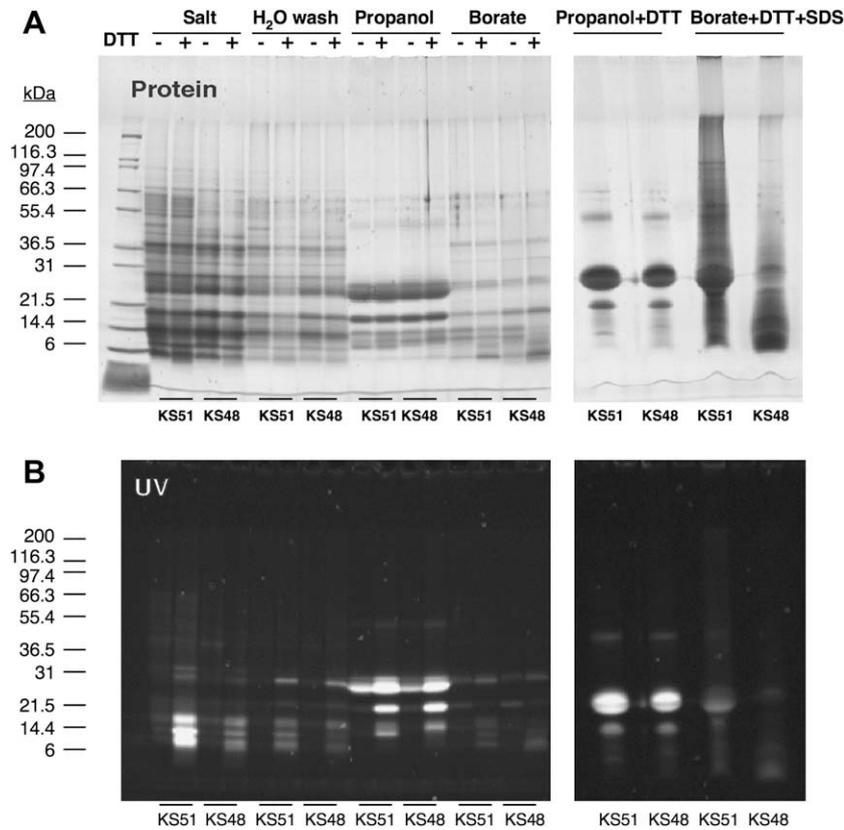


Fig. 1. Effect of DTT on redox status of protein fractions extracted from grain of KS48 and KS51. (A): Protein stained with Coomassie blue; (B): protein labeled with mBBr visualized by UV absorption. Tris–HCl gel was used. Individual protein fractions are identified above (A).

the relative abundance of *intermolecular* S–S groups in the cross-linked glutelin fraction in KS51 suggests that the non-kafrins, which make up the protein matrix, have a strong influence on the digestibility of KS51 relative to KS48.

3.3. Time course of *in vitro* pepsin digestion

The gel-based system used to analyze protein digestion by pepsin (Aboubacar et al., 2001; Nunes et al., 2004) requires less material, is faster and can accommodate more samples than a commonly used batch procedure (Mertz et al., 1984). The gel procedure not only reveals the types of undigested kafrins, but also

allows an estimate of the percentage digested over time. This gel system has been successfully used to separate sorghum proteins (Bean, 2003) and wheat glutenins (Kasarda et al., 1998). In our study, we observed that separation of different kafrin types is improved in the neutral pH environment of the NuPAGE Novex Bis–Tris gels relative to that seen with the conventional Tris–HCl gels (cf. Fig. 2A vs. Fig. 1A). A NuPAGE gel image (Fig. 2A) shows that high M_r components of the insoluble protein fraction, including glutenins (35–100 kDa), were readily digested, while the smaller kafrins (M_r 18–27 kDa) were more resistant. Further, the time course of the *in vitro* digestion of this fraction demonstrated that kafrins in KS48 were digested more rapidly by pepsin than those in KS51 (Fig. 2A).

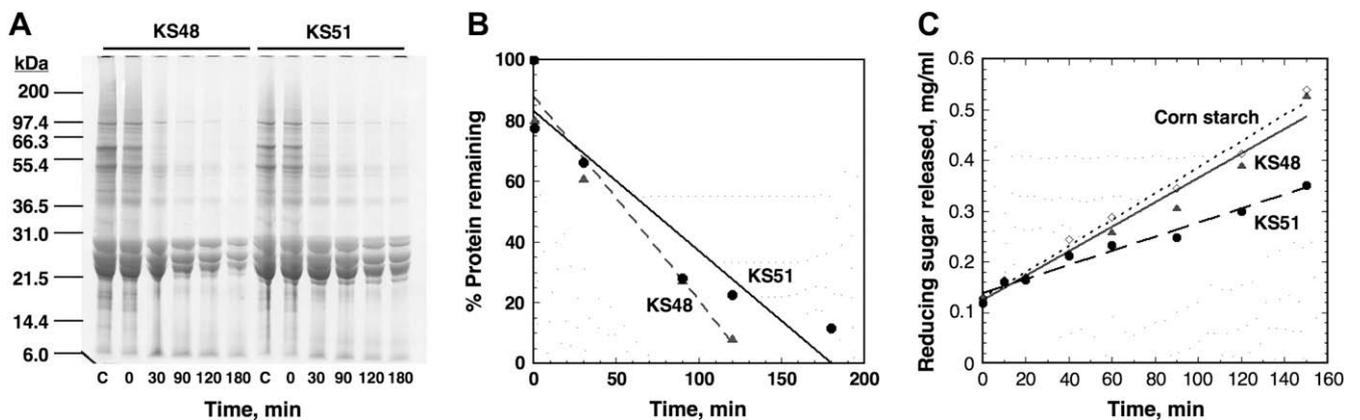


Fig. 2. Time course of *in vitro* digestion of storage protein and starch in KS48 and KS51. (A): BT–NuPAGE gel showing digestion of glutelins and kafrins over time; (B): estimation of glutelin and kafrin digestion rate with the NI protein assay. Results were calculated by linear regression and expressed as % of control without pepsin; (C): starch digestion by bacterial α -amylase. Corn starch is shown as a control.

Gel densitometry measurements revealed that various kafirins, α , β and γ (El Nour et al., 1998), were digested at different rates: $\beta > \gamma > \alpha$ (data not shown). Furthermore, data on the disappearance with time of insoluble proteins (kafirins and glutelins) (Fig. 2B) indicated that KS48 was digested more rapidly than KS51 – negative slope of 0.643 (SE \pm 0.010) for KS48 vs. 0.530 (SE \pm 0.023) for KS51, mean of three determinations. These values, which represent a 22% rate difference, positively correlate with the corresponding IVDMD difference of 36%. The lower value obtained with the *in vitro* pepsin method could be due to use of a single protease vs. a mixture of proteolytic and amylolytic enzymes present in ruminal fluid used in the IVDMD procedure.

3.4. Time course of *in vitro* starch digestion by α -amylase

The finding that the 12-h IVDMD value was higher for KS48 than KS51 suggested that starch digestion alone might follow the same trend, and this was confirmed (Fig. 2C). The average of three time-course experiments for *in vitro* digestion of meal from KS48 and KS51 by bacterial α -amylase gave slopes of 1.74×10^{-3} (SE 1.69×10^{-4}) and 1.41×10^{-3} (SE 5.01×10^{-5}), respectively. These results represent a 23% difference in starch digestion rate between the two lines – a value similar to that obtained with the *in vitro* pepsin (Fig. 2B) and IVDMD (Pedersen et al., 2000) procedures. More significantly, the three independent *in vitro* techniques used to assess digestibility showed a positive correlation. This is the first time the three approaches have been compared in a single study.

Ezeogu et al. (2005) made significant contributions to the idea that *in vitro* digestibility of starch in sorghum and maize flours is related to endosperm texture and cooking conditions. These researchers found that starch digestion was significantly higher in floury sorghum endosperm than in vitreous endosperm, while their counterparts in maize were similar in this regard. Cooking with ME increased starch digestion in both sorghum and maize, but more so with sorghum, and with vitreous endosperm flours. This is consistent with the fact that more polymeric kafirins, formed by intermolecular disulfide bonds (Chandrashekar and Mazhar, 1999; Oria et al., 1995a), occurred in the vitreous endosperm fraction (Ezeogu et al., 2005; Kumari and Chandrashekar, 1994), probably because the cysteine-rich γ - and β -kafirins abundant in that part of the sorghum grain (Chandrashekar and Mazhar, 1999; Ezeogu et al., 2005, 2008; Mazhar and Chandrashekar, 1995). Unlike their findings, which were based on similar types of starch – *i.e.*, a 75:25 amylopectin/amylose ratio from different parts of the sorghum and maize endosperm – our results are based on different types of

starch, *i.e.*, an amylopectin/amylose ratio of 97:3 and 70:30, for KS48 and KS51, respectively (see below).

3.5. Starch digestibility differences in starch in KS48 and KS51

In the original rapid protein digestion assay (Aboubacar et al., 2001), the pellet remaining after extraction of kafirins and glutelins with borate–SDS–ME was not further analyzed. In this study, we attempted to fill this gap by analyzing the residual pellet that still contained between 2.2–2.5% (pepsin-treated) and 4.7–5.0% (buffer control) of protein nitrogen (data not shown). After boiling the residual pellet with excess borate–SDS–ME buffer, we unexpectedly observed a marked difference in the gelatinization properties of starch in KS48 and KS51. The KS48 pellet yielded a transparent, soft starch gel that swelled to a greater extent than the KS51 counterpart. Further, when equal amounts of extraction buffer were added to the pellets before boiling, only a small amount of supernate was recovered after centrifuging the cooled gel KS48 preparation, whereas KS51 yielded a larger amount of clear supernate that separated easily from the harder opaque gel (data not shown). The gelatinization differences in KS48 and KS51 starch resemble those observed for waxy vs. non-waxy starch.

In addition to obvious swelling differences in gelatinized starch, supernates from KS48 and KS51 contained different proteins. One-dimensional SDS-PAGE analysis revealed a major protein band of $M_r \sim 58$ kDa in KS51, not detected in KS48 (Fig. 3A). Because it was released from starch granules only after gelatinization, the protein did not appear to change after treating the parent meal with pepsin (data not shown). Two-dimensional gel analysis gave information on the nature of the unknown band (Fig. 3C, D). Two major 61 kDa spots with acidic pI's, uniquely observed in KS51 (note arrows, Fig. 3D), were identified as granule-bound starch synthase I by mass spectrometry [GBSSI, chloroplast (sorghum) accession No. Q43134]. Identity was further confirmed by Western blot analysis using an antibody against maize waxy protein (Fig. 3B). In KS51, a highly antibody-reactive band was seen at 58 kDa and a less reactive one at 61 kDa, corresponding to two isoforms of GBSSI: a major form in the endosperm (58 kDa) and a minor form in the pericarp (61 kDa) (Nakamura et al., 1998). While lower levels of the pericarp isoform were seen in KS48 relative to KS51, the endosperm component was not detected. The granule-bound isoform of starch synthase, GBSSI, also referred to as the waxy protein encoded in the waxy (*Wx*) locus of cereals, functions specifically to elongate amylose (Denyer et al., 2001) by adding glucose residues in α -1,4-glycosidic

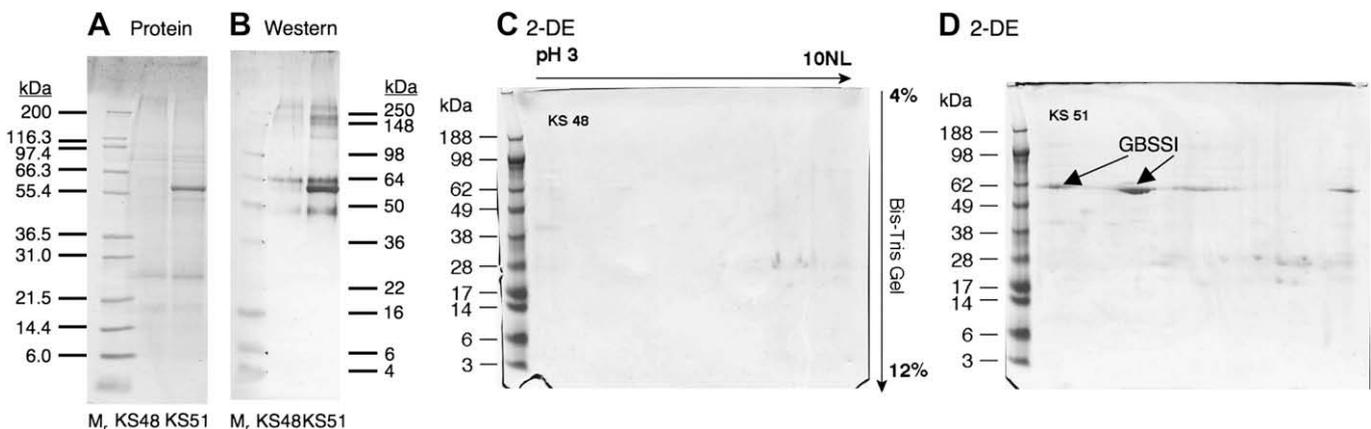


Fig. 3. Starch-bound proteins extracted from KS48 and KS51. The pellet remaining after removing glutelins and kafirins was boiled with borate–SDS–2-ME buffer and the proteins solubilized were analyzed by one-dimensional SDS-PAGE. (A): Protein; (B): Western blot analysis using waxy protein antibody; (C and D): two-dimensional gel analysis of starch-bound proteins extracted in (A).

linkage. Recent genetic experiments have revealed that sorghum contains at least two GBSS genes (Pedersen et al., 2005).

3.6. Starch of waxy and non-waxy sorghum

Normal or non-waxy sorghum starch has approximately 75% amylopectin and 25% amylose, while waxy starch contains nearly 100% amylopectin (Ezeogu et al., 2005; Rooney and Miller, 1982; Rooney and Pflugfelder, 1986). The apparent absence of GBSSI protein in the endosperm of KS48 (Fig. 3A, B) indicates that its starch should be low in amylose and contain mainly amylopectin.

Waxy and non-waxy polymers of starch can be distinguished by iodine staining (Denyer et al., 2001; Pedersen et al., 2004). Starch of non-waxy lines, containing amylose and amylopectin, forms blue-black complexes with iodine, while starch from waxy lines, lacking amylose, stains reddish brown. Since the waxy nature of KS48 and KS51 was not indicated in the USDA-ARS, Genetic Resource Information Network (Pedersen et al., 2004), grains from the two lines were stained with iodine. Results confirmed KS48 has a waxy (brownish red) and KS51 a non-waxy (dark blue) type of endosperm (see below). Biochemical analyses confirmed this observation: KS48 contains 3% and KS51 30% amylose.

Numerous studies have shown that starch is more readily digested in waxy sorghum than in non-waxy types [for review, see (Rooney and Pflugfelder, 1986)]. Our results are consistent with this conclusion, not only for starch but also for protein (KS48 > KS51). Digestibility of protein, however, may not necessarily be consistent with waxy vs. non-waxy sorghum lines. The nature of the protein matrix and the extent of embedded starch in the endosperm are proposed to account for this inconsistency (Chandrashekar and Kirleis, 1988; Duodu et al., 2003; Rooney and Miller, 1982; Rooney and Pflugfelder, 1986; Shull et al., 1990). To gain insight into this point, we conducted experiments with our waxy and non-waxy lines as described below.

3.7. Sequential addition of α -amylase and pepsin

Digestion protocols, using sequential addition of pepsin followed by α -amylase, and vice versa, were designed to study the inter-relationship of starch and protein in KS48 and KS51. Prior digestion with pepsin, followed by treatment with human saliva α -amylase (designated P/A in Table 2), enhanced to a greater extent starch hydrolysis, relative to treatment with α -amylase followed by pepsin (A/P), in KS48 compared to KS51 (1.8- vs. 1.5-fold, respectively). This observation suggests that KS51 starch granules were intrinsically harder to digest than those of KS48, even if the protein matrix was partially removed by prior pepsin digestion. This explanation agrees with the time course of *in vitro* starch digestion of meal (Fig. 2C), as well as with the relative ease of digestion of isolated waxy vs. non-waxy starch (Rooney and Miller, 1982; Rooney and Pflugfelder, 1986).

Reversal of this protocol—removal of starch prior to pepsin digestion—showed the opposite effect; proteolysis was enhanced to a greater extent with KS51 than KS48 (2.2- vs. 1.6-fold) (Table 3).

Table 2
Starch digestion with and without prior removal of protein by pepsin

| Sorghum line | With pepsin pre-treatment (pepsin/ α -amylase ^a) | Without pepsin pre-treatment (α -amylase/pepsin ^a) | Ratio P/A:A/P |
|--------------|---|--|---------------|
| | mg reducing sugar/h | mg reducing sugar/h | |
| KS48 | 84.8 ± 4.2 | 47.6 ± 2.1 | 1.8 |
| KS51 | 59.1 ± 2.7 | 39.3 ± 1.3 | 1.5 |

^a Mean of 3 experiments.

Table 3
Protein digestion by pepsin without and with prior removal of starch by α -amylase

| Sorghum line | With α -amylase pre-treatment (α -amylase/pepsin ^a) | Without α -amylase pre-treatment (pepsin/ α -amylase ^a) | Ratio A/P:P/A |
|--------------|--|---|---------------|
| | Protein remaining, volume, % | Protein remaining, volume, % | |
| KS48 | 65.4 ± 2.0 | 40.9 ± 2.7 | 1.6 |
| KS51 | 75.3 ± 1.5 | 34.0 ± 3.8 | 2.2 |

^a Mean of 3 experiments.

This unexpected result suggests that, in addition to the intrinsic properties of the starch, spatial arrangement between the granules and protein bodies differs in the two lines. Further, starch in KS51 appears not only to be harder to digest with α -amylase, but also more capable of impeding protein digestion by pepsin than in KS48. Once starch-imposed restrictions are removed, protein in KS51 appears more exposed and thus more accessible to pepsin. A question arises as to whether the observed difference could simply be due to the physical properties of the two grains, *i.e.*, variation in the hardness of the two grains, or different ratios of hard (closed) to soft (open) endosperm, as reported by Ezeogu et al., 2005. Hardness data for KS48 and KS51 samples using the single kernel characterization system (SKCS) showed that they are practically and statistically the same, with a mean for seed hardness of 69.12 and 69.83, respectively. Therefore, our interpretation is that the difference observed above is mostly due to the properties of the starch and proteins.

3.8. Effect of DTT reduction on digestion of kafirins and glutelins by pepsin

Reducing agents, such as 2-ME (Elkhalifa et al., 1999; Ezeogu et al., 2005, 2008; Hamaker et al., 1987) and DTT or thioredoxin (del Val et al., 1999), were shown to improve protein digestibility, thus highlighting the importance of components with disulfide bonds (Hamaker et al., 1987). In assessing DTT effects, we observed that 42% and 50% of the kafirins of KS48 and KS51, respectively, were not digested by pepsin in control samples. Reduction with DTT

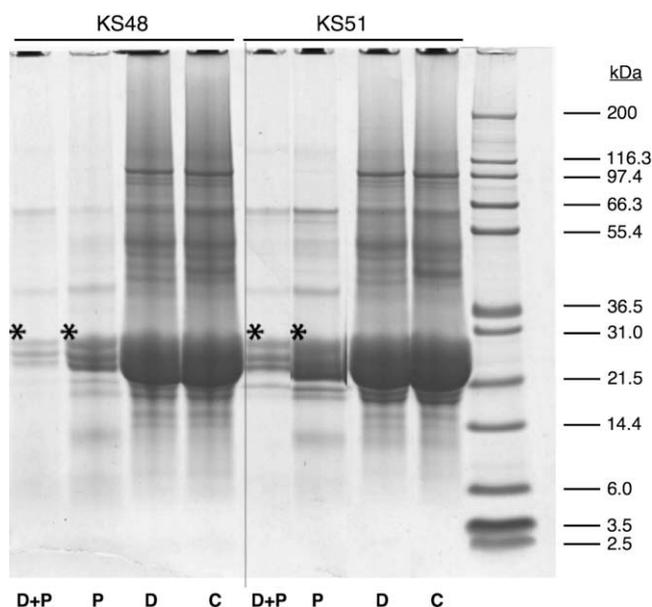


Fig. 4. Effect of redox status on digestion of the combined glutelin and kafirin fractions of KS48 and KS51 by pepsin. D + P = DTT + pepsin; P = pepsin; D = DTT; C = control (minus DTT and pepsin). * = Kafirin region.

enhanced digestibility by 3.5-fold and 2.0-fold, with undigested protein decreasing from 42% to 12% for KS48 and from 50% to 25% for KS51 (Fig. 4, * designates kafirin region). To achieve a level of kafirin digestion with KS51 equivalent to that of KS48, either a longer incubation period or more reductant was required (data not shown).

3.9. Microscopy studies

Our results thus far show that a greater abundance of disulfide proteins and the non-waxy type of starch contribute to the low digestibility of KS51 vs. KS48. We have performed experiments with additional waxy and non-waxy lines of sorghum and corn (3 pairs of sorghum lines, 1 pair of corn lines). Digestion of waxy starch was consistently faster than that of non-waxy starch in all samples tested but protein digestibility was mixed (data not shown). Thus, this data also show the importance of the interactions between starch and protein to the digestibility of both

components. However, they provide no information on the nature of the differences observed in these interactions between the two lines.

To study this point, we examined iodine-stained sections of grains microscopically and found that the endosperm in KS51 was more highly organized than that of KS48—i.e., there was a distinct demarcation between floury and corneous endosperm in KS51, not present in KS48 (Fig. 5A). Taken together, the analytical data presented above and the iodine staining results confirmed the earlier classification of the lines as waxy (KS48) and non-waxy (KS51) (Pedersen et al., 2004, 2005). Electron micrographs revealed organizational differences between the two. While floury endosperm was similar (Fig. 5C), corneous endosperm differed markedly in the two lines (Fig. 5B). Protein bodies were more numerous and more tightly associated with starch granules in KS51 than in KS48 (Fig. 5B). This type of interaction between the two components could contribute to the low digestibility of protein and starch in KS51.

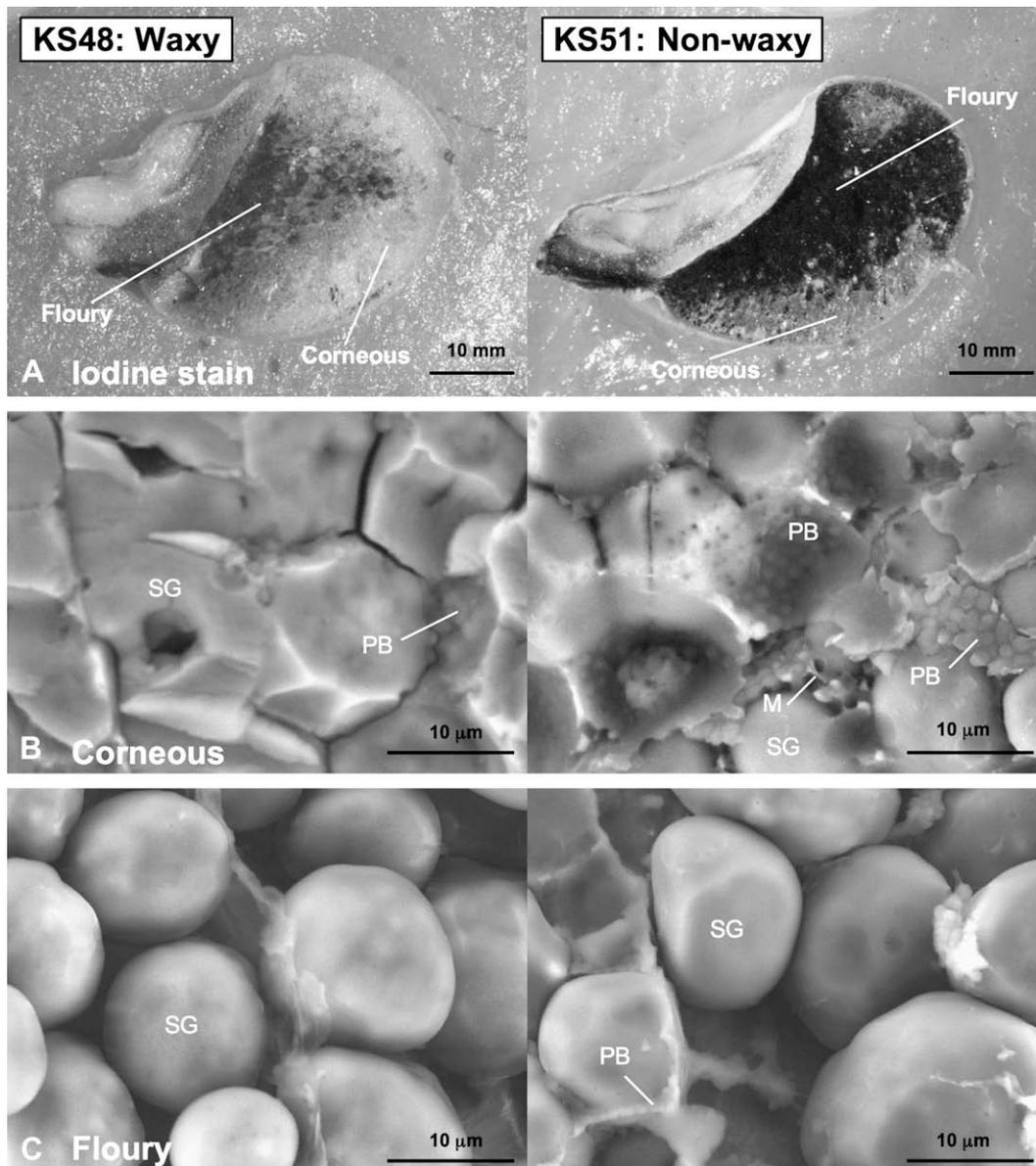


Fig. 5. Light and electron microscopy of KS48 and KS51 seeds. A. Cross section of seeds stained with iodine showing floury (F) and corneous (C) endosperm. Magnification: 32 \times . B. Electron micrograph of corneous endosperm of KS48 and KS51, 5000 \times . C. Electron micrograph of floury endosperm of KS48 and KS51, 5000 \times . SG = starch granule; PB = protein body; M = protein matrix.

In summary, our results highlight the importance of disulfide proteins and GBSSI in conferring the chemical and structural properties that influence the digestibility of stored reserves, both protein and starch. These findings also add insight into the role of the protein matrix and its interaction with starch. Comparison of two sorghum lines with a common pedigree that differed in digestibility made it possible to demonstrate how protein–starch interactions in the seed influence the digestibility of each. Attempts to improve the digestibility of grain by classical breeding and genetic engineering should further our understanding of these factors and their relative contribution to digestibility. These efforts may also provide answers to related fundamental questions, such as what role the key redox protein, thioredoxin, plays in forming and maintaining the protein matrix (Buchanan and Balmer, 2005) and the importance of this unique packaging of stored reserves to the structure and physiology of seed. Answers to these questions will in all likelihood be relevant to human and animal nutrition and to the use of sorghum as a biofuel.

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