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Qualitative assessment of 'highly digestible' protein mutation in hard endosperm sorghum and its functional properties

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ABSTRACT

Sorghum mutants with altered protein body structure have improved protein nutritional quality; however, practical methods to accurately track heritability of the trait are lacking. We evaluated suitability of the *in vitro* pepsin assay, and a new high-resolution field emission electron microscopy (FE-SEM) method to detect the mutation (HD) in hard-endosperm sorghum; and compared the physicochemical properties of experimental HD sorghums to wild type (LD) lines. FE-SEM reliably resolved sorghum protein body structure, allowing for qualitative classification of sorghum as HD or LD. The pepsin assay was less reliable, with significant variations across environments. Nevertheless, HD lines averaged higher protein digestibility (69.4% raw, 57.6% cooked) than LD lines (61.7% raw, 45.6% cooked). The HD lines also had better water solubility and starch pasting profiles than LD lines. FE-SEM, but not pepsin assay, reliably detects HD nutation in sorghum. The HD trait may improve food-use functionality of sorghum.

1. Introduction

Grain Sorghum (*Sorghum bicolor* L. Moench) is an important food crop in arid and semi-arid areas of Africa, Asia and Latin America. As a food grain, interest in sorghum use in the developed world is growing due to dietary diversification trends like 'gluten-free' and 'ancient grains'(Awika, 2017), as well as its increased recognition as a health-promoting grain with unique bioactive compounds that contribute to reduced inflammation and adiposity, postprandial blood glucose response, and other chronic conditions (Agah, Kim, Mertens-Talcott, & Awika, 2017; Arbex et al., 2018; Simnadis, Tapsell, & Beck, 2016; Yang, Allred, Dykes, Allred, & Awika, 2015). However, application of sorghum in modern food processing is challenging relative to other cereal grains.

Starch granules in the sorghum corneous endosperm are surrounded by a matrix of hydrophobic kafirin protein bodies (Belton, Delgadillo, Halford, & Shewry, 2006); this reduces the extent of granule swelling and starch availability to interact with other ingredients during processing to produce desirable food texture (Taylor & Emmambux, 2010; Wong et al., 2009). In addition, during cooking, the β- and γ-kafirins at the periphery of the protein bodies extensively cross-link via disulfide linkages, further inhibiting the ability of starch to swell (Duodu et al., 2002; Taylor & Emmambux, 2010). The restricted starch functionality results in food products that have a sandy/gritty, dense, crumbly, or firm texture. Additionally, due to the extensive cross-linking, cooked sorghum proteins generally have reduced digestibility compared to other cereal grain proteins (Aboubacar, Axtell, Huang, & Hamaker, 2001; Duodu, Taylor, Belton, & Hamaker, 2003; Oria, Hamaker, & Shull, 1995). This is an important issue, because areas where sorghum is consumed as a staple generally have prevalence of protein malnutrition (Batool, Butt, Sultan, Saeed, & Naz, 2015).

Sorghum mutants with irregularly shaped protein bodies and soft endosperm have increased protein digestibility, and are thus commonly referred to as high digestible protein (HD) sorghum (Oria, Hamaker, Axtell, & Huang, 2000). These sorghum mutants may also have enhanced functionality in applications relevant to food, malting, and bioethanol production (Da Silva, Taylor, & Taylor, 2011; Elhassan, Naushad Emmambux, Hays, Peterson, & Taylor, 2015; Mezgebe, Abegaz, & Taylor, 2018; Oria et al., 2000; Weaver, Hamaker, & Axtell, 1998; Winn, Mason, Robbins, Rooney, & Hays, 2009). The HD mutation in sorghum is also associated with enhanced protein quality (increased lysine content) (Weaver et al., 1998) and could thus benefit protein malnutrition in populations that consume sorghum as a staple, in addition to making sorghum more appealing to the feed industry. However, the soft endosperm trait is undesirable as it is negatively associated with agronomic performance (susceptible to weathering) and grain handling and processing (Rooney & Pflugfelder, 1986). Consequently, genetic improvement efforts to introduce the HD trait in

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agronomically competitive hard endosperm sorghum are underway (Jampala, Rooney, Peterson, Bean, & Hays, 2012; Winn et al., 2009). Thus practical methods that can reliably detect the HD trait in sorghum are necessary.

The standard (Mertz et al., 1984) or rapid (Aboubacar et al., 2001) *in vitro* pepsin assays are the most widely used technique to determine protein digestibility of grain sorghum, and consequently indirectly identify the HD trait. However, whether these quantitative methods can accurately identify the trait in improved hard endosperm sorghum is unknown. For definitive determination of the mutation, transmission electron microscopy (TEM) is the standard method to identify the HD protein body structure (Oria et al., 2000, 1995). Unfortunately, TEM requires a time consuming and tedious procedure (Oria et al., 2000) which involves fixing, staining, and gradual dehydration. Thus, it is not useful for screening significant numbers of samples that would be necessary in genetic improvement programs. In this regard, there remains a need for a practical qualitative method to readily identify the HD mutation in sorghum and help in assessing the heritability of the trait in sorghum breeding. We hypothesized that the *in vitro* pepsin assay cannot reliably identify HD mutation in hard endosperm sorghum due to confounding effect of endosperm architecture among other variables.

Our goal in this research was thus twofold: first, we aimed to establish the suitability of the pepsin assay, and a high-resolution field emission scanning electron microscopy (FE-SEM) as a new qualitative method to identify the HD mutation in genetically related hard endosperm sorghum genotypes known to segregate for the trait; and then identify how the HD mutation affects physicochemical properties relevant to food processing and quality in sorghum of varying starch composition.

2. Materials and methods

2.1. Sample description and preparation

The samples used in this study included 86 experimental lines under development by Texas A&M University to transfer the HD trait into commercially competitive hard endosperm sorghum with normal and waxy starch traits. A soft endosperm sorghum line known to have the protein body mutation (P850029) (Weaver et al., 1998) was used as HD control, whereas two commercial food sorghums were used as normal protein (LD) sorghum. Samples were grown in 2014 and 2015 crop years in College Station and Halfway, Texas. The samples were stored at −20 °C until analyzed. Prior to analysis, samples were cleaned and milled (UDY Cyclone sample mill model: 3010–014, Fort Collins, USA) to pass through a 1 mm screen size. Pepsin enzyme powder (EC 3.4.23.1, lot number: SLBP2152V, Activity ≥ 250 units/mg) was obtained from Sigma Aldrich.

2.2. Grain physical parameters

The kernel hardness and weight were assessed using a single kernel characterization system (SKCS 4100, Perten Instruments, Springfield, Illinois) as described by Pedersen et al. (1996). The system records kernel hardness, seed diameter and weight for 300 individual kernels and reports the average values. Grain density was analyzed using a gas displacement multi pycnometer (MVP-1, Quanta Chrome). Rapid Iodine staining technique was used to identify waxy phenotypes.

2.3. In vitro pepsin assay

In vitro protein digestibility was investigated using rapid pepsin assay as described by Mertz et al. (1984) and modified by Aboubacar et al. (2001). Ground samples (200 mg, ≤1 mm particle size) for raw digestibility and wet cooked flour samples (1 mL water added, cooked at 95 °C for 25 min, then cooled to room temperature) for cooked digestibility were suspended in 35 mL pepsin solution(Mertz et al., 1984) (1.5 mg/mL of phosphate buffer at pH = 2) and the suspension was incubated in a gently shaking water bath at 37 °C for 2 h. Immediately after incubation, the enzyme activity was stopped by changing the pH of the system with addition of 2 mL 2 N NaOH. The suspension was then centrifuged at 4900*g*/4°C for 20 min. The residue was washed twice in phosphate buffer (pH = 7) and then dried in a forced air oven at 40 °C for 24 h. Nitrogen contents of raw flours and the digested residue were determined using LECO combustion method as described by Sweeny (Sweeney, 1989). A factor of 6.25 was used to estimate protein content. Protein digestibility was calculated as:

$$Digestibility\,(\%) = \left(\frac{P_{Samples} - P_{Residue}}{P_{Samples}}\right) \times 100,$$

where P = protein content.

Data was corrected for moisture content and reported on dry basis.

2.4. Field emission scanning electron microscope (FE-SEM) imaging

Protein bodies were initially isolated from the control HD and normal (LD) sorghum lines using the wet milling method described by Buffo, Weller, & Parkhurst (1998) for scanning electron microscopy (SEM) (with modifications) to check if the HD protein body structure could be distinguished. Samples (25 g) were soaked in a steeping solution (2.22 g sodium bisulfite and 4.17 mL lactic acid in 1000 mL water) for 48 h at 50 °C. The steeping water was drained and the seeds rinsed in distilled water. The seeds (in 25 mL water) were then pulverized using a coffee grinder to release the endosperm starch and proteins. The slurry was screened through 63 μm mesh to remove germ and bran. The endosperm slurry was then centrifuged at 7155*g* for 10 min at 8 °C. The protein bodies formed a visible thin film on top of the starch after the supernatant was carefully discarded. The protein bodies were gently scrapped off, transferred to aluminum tins and diluted in distilled water to form a thin layer for uniform drying at 40 °C in a forced air oven for 24 h. The dried mass was carefully scraped and used for imaging. Environmental SEM imaging was initially tested, but did not produce adequate resolution to distinguish between HD and LD protein body morphology. FE-SEM was subsequently used.

For the FE-SEM, the isolated protein body powder was sputter-coated (5 nm thickness) with a mixture of platinum (80%) and palladium (20%) to improve the conductivity of the sample surface. The samples were then loaded into the vacuum chamber of the FE-SEM, (Model JEOL JSM-7500F, Japan). The pressure in the specimen chamber was maintained below 3.6×10^{-4} Pa. The region of interest of the endosperm was selected under low magnification (LM) and the desired scanning/imaging of the morphology were done using the SEM mode in the range of ≤1 μm. The protein bodies were imaged and compared.

Based on the successful resolution of the isolated protein bodies by FE-SEM, whole seed endosperm samples were imaged in situ. Dried seeds were freeze-fractured to obtain clean cleavage of the endosperm morphology and to avoid damages to the starch and protein structures. This was achieved by keeping the seed in liquid nitrogen for 3 min and gently cracking it using a small mortar and pestle. One-half of the cracked seed was then mounted on a sample holder using double sticky carbon tape and sputter-coated similar to that for the protein isolates. In instances of excessive static charging, scanning was done in freeze-mode to obtain good quality images. Thirty-six samples selected from the experimental lines to represent the observed variations in digestibility and starch composition were selected for imaging. The method was used to qualitatively classify the experimental sorghum samples as HD (with protein body mutation) or LD (wild type).

2.5. Lysine content

Lysine is the most limiting amino acids in sorghum (Virupaksha &