SORGHUM GRAIN CHEMISTRY AND FUNCTIONALITY: EFFECTS OF KERNEL MATURITY, GENETIC, ENVIRONMENTAL AND MANAGEMENT FACTORS.

by

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B.S., Kansas State University, 2003
M.S., Kansas State University, 2005

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

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Abstract

Sorghum (*Sorghum bicolor* [L.] Moench) is the fifth most important cereal grain grown in the world. Sorghum is an important cereal crop for both animal feed and biofuel production in the United States. The genetic, environmental, and agronomic management influences on sorghum starch and protein chemistry and functionality were evaluated. A method was developed to determine amylose content in cereal starches that achieved the same level of accuracy and precision as traditional methods, but had the capability of analyzing 50 samples per day or approximately a 10-fold increase in throughput. The effect of kernel maturity on sorghum starch properties was conducted by collecting grain from two hybrids at various stages throughout kernel development. The samples ranged from 16.3% amylose in 10 days after anthesis (DAA) to 23.3% amylose in 35 DAA. Starch thermal properties were also altered due to DAA, most notably the ΔH was 16.1 J/g at 14 DAA and 9.45 J/g at 56 DAA. In a separate study using the same developmental samples the protein and starch digestibility was analyzed. The kernel maturity had a notable effect on digestibility with the maximum digestibility occurring at 17 DAA with 82.44% digestible protein. In another study a diverse set of 19 sorghums was grown in three locations in Kansas to evaluate the genetic, location, and genetic x location effect on grain quality attributes. The physical and chemical properties of the sorghums were greatly affected by the genotype, environment, and the GxE interaction. Protein content ranged from 11.09% to 15.17% and digestibility ranged from 45.58% to 62.05% due to genotype. The final study investigates the role of agronomic management on sorghum grain quality. A sorghum hybrid was grown on plots with varying nitrogen fertilization rates and cover cropping systems that are currently used by Kansas producers. Grain attributes such as hardness and size were variable due to the treatments but negative impacts to protein digestibility were not seen due to cropping system. Sorghum grain quality is affected by many variables and a better understanding of the variables will lead to a higher quality product.

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Preface

The chapters in this dissertation were written to be manuscripts submitted for publication, therefore references are included for each chapter and formatted for the potential submission according to journal guidelines.

Chapter 1 - Introduction

Starch is produced in the tissues of green plants to serve as an energy source and an energy reserve. In cereal grains, starch is stored in the developing seed to serve as the source of energy for the embryo to develop into the next generation. Starches from cereal grains and other plant sources have been utilized by humans for thousands of years as a food source as well as other industrial uses. A complex enzymatic pathway is used by the plants to convert sugars produced by photosynthesis to a water-insoluble starch granule. Starch granules are composed of two types of glucose homopolymers, amylose and amylopectin. Amylopectin, a large branched polymer, is the major component of the starch granule contributing approximately 70-80% of the total starch by weight. Amylose is a smaller, nearly linear polymer that contributes the remaining 20-30% of starch weight.

This review will focus on the biosynthesis of starch in cereal grains by examining the enzymes responsible for starch synthesis and the formation and development of the starch granule architecture. The review will also address the compositional and functionality changes of starch throughout seed development along with key environmental influences. An examination of the monosaccharide, primarily glucose, separation and detection by HPLC will also be discussed due to glucose's relevance when analyzing for starch digestibility.

Enzymatic Pathway

There are several different enzymes that are vital to the development of the starch granule. Each enzyme has a specific function, beginning with the formation of ADP-glucose and ending with final trimming of the starch chains.

ADP-glucose pyrophosphorylase (AGPase)

The first step in the synthesis of starch is the production of ADP-Glucose. AGPases (E.C. 2.7.7.27) produce ADP-Glucose and pyrophosphate from Glucose-1-phosphate and ATP. AGPases are comprised of two small subunits and two large subunits that are coded by the genes *shrunken2* and *brittle2* in maize (Hannah and Nelson, 1976). The small subunits are primarily responsible for the catalytic function of the enzyme while the large subunits are regulatory for allosteric effects (Cross et al., 2005). Recently it has been determined that most (approximately 65-95%) of the activity of AGPases are found in the cytosol and not in plastids as in other plant species (Beckles et al., 2001; Comparot-Moss and Denyer, 2009). AGPases are activated by 3-phosphoglycerate (3-PGA) and inhibited by inorganic phosphate (Pi) (Cross et al., 2004).

Environmental factors such as high temperature are known to regulate endosperm AGPas (Greene and Hannah, 1998). Mutants identified to be more heat stable or resistant to inhibition have produced increases in starch production in both wheat and rice (Smidansky et al., 2002; Smidansky et al., 2003), which ultimately leads to increased grain yield.

Starch Synthases (SSs)

Starch synthases (E.C. 2.4.1.21) are found in five main groups; granule bound (GBSS), SSI, SSII, SSIII, and SSIV. The SSs are responsible for the elongation of linear glucan chains and ultimately the synthesis of both amylose and amylopectin. The elongation is the result of catalyzing a new α-(1,4) linkage between an existing glucan or glucan chain and a new ADP-Glucose produced by the AGPases. Historically the prevailing opinion was that the addition occurs at the non-reducing end of the linear chain, but recent studies have suggested that the addition occurs at the reducing end (Mukerjea et al., 2002; Mukerjea and Robyt, 2005). The different isoforms of SSs are responsible for addition to different chain lengths and amylose or amylopectin synthesis.

GBSS is the enzyme responsible for the production of the long linear chains found in amylose and exists in two isoforms. The GBSSI isoform is most commonly found in the endosperm tissue whereas GBSSII is typically found in non-storage tissue (Nakamura et al., 1998; Vrinten and Natamura, 2000). Mutations to the *Waxy* gene encoding GBSS result in the starch with reduced or absent amylose component without affecting the total starch content of the cereal grain (Tsai, 1974; Fujita et al, 2001).

The soluble SSs produce the glucan chains that comprise the amylopectin molecule. The SS isoforms have a preferential chain length in which they elongate, but there is a slight overlap (Zhang et al., 2008). SSI is responsible for the shortest glucan chains that typically have a degree of polymerization (DP) of 10 or less (Commuri and Keeling, 2001), and are expressed early in grain development (Peng et al., 2001). SSII appears to synthesize glucan chains of DP 12-24 by elongating the short chains (Fontaine et al., 1993). The *sugary2* mutation causes a lack of SSII which results in an increase in DP 6-11chains and a decrease in DP 13-20 chains (Zhang et al., 2004). The loss of function mutants of SSIII result in a decrease in chains longer than 30 (James and Myers, 2009). The SSIII has been linked to the *dull1* mutant in maize (Gao et al., 1998) and exhibits changes in granule morphology and crystallinity (Fujita et al., 2007). SSIV is

the most recently discovered SS (Dian et al., 2005). SSIV role in chain length has not been fully determined but it has been linked along with SSIII to a role in granule initiation in *Arabidopsis* (Szydlowski et al., 2009).

Starch Branching Enzymes (SBEs)

The SBEs (E.C. 2.4.1.18) form the branched structure of the amylopectin molecule by cleaving the α-(1,4) linkages and transferring the reducing ends to a α-(1,6) branch point (Guan et al., 1997). There are two classes of SBEs, SBEI and SBEII, which are distinguished by the glucan chain length that they produce. SBEI produces longer chains (DP ≥ 16) whereas SBEII prefers shorter chains (DP ≤ 12) (Guan et al., 1997). SBEII appears to have a greater affinity towards amylopectin and SBEI exhibits higher branching rates in amylose (Guan and Preiss, 1993; Takeda et al., 1993). SBEII has two isoforms which are found in different cereals, SBEIIa in wheat and barley, SBEIIb in maize and rice (Regina et al., 2010). The *amylose-extender* mutation is linked to the gene encoding for SBEII and produces a high-amylose starch (Nishi et al., 2001).

Starch Debranching Enzymes (DBEs)

Two types of DBEs are found in the cereal endosperm, isoamylase (E.C. 3.2.1.68) and pullulanase (E.C. 3.2.1.41). These two enzymes hydrolyze the α-(1,6) linkages in the glucan chains and are thought to be important to the formation of crystalline amylopectin. The mutants that are deficient for isoamylase or *sugary1* gene exhibited an increase in sugars and a water-soluble polysaccharide known as phytoglycogen (James et al., 1995). The role of the DBEs in starch synthesis is not fully understood therefore several models have been proposed to explain the DBE functionality. One model is the glucan-trimming model, which states that the DBEs remove improperly positioned branches that limit the formation of double helices and ultimately the dense packing into the insoluble granule (Ball et al., 1996; Myers et al., 2000). Another model proposes that the DBEs are removing soluble glucans from the stroma limiting the random synthesis by SSs and SBEs resulting in less accumulation of phytoglycogen (Zeeman et al, 1998).

Miscellaneous Enzymes

A few other enzymes are also associated with the synthesis of starch. Starch phosphorylase (SP) (E.C. 2.4.1.1) and amylomaltase (D-enzyme) (E.C. 2.4.1.25) roles are not fully understood in starch biosynthesis but mutants lacking the respective enzymes have drastic effects on starch content and structure, such as altered amylopectin chain length distribution (Bresolin et al., 2006; Satoh et al., 2008).

The Starch Granule

After the glucan polymers are synthesized they are bundled into discreet water insoluble granules. The granules are produced in cellular organelles referred to as the amyloplasts, which in most cereal grains produce one granule per amyloplast. However, in rice and oat many very small granules are produced in one amyloplast resulting in compound granules. The entire process of starch granule formation is not fully understood, but there has been research to suggest that the process is more physical than biological. Research using X-ray scattering and NMR have demonstrated that amylopectin may be structured as a side-chain liquid crystalline polymer (Waigh et al., 1998; Waigh et al., 2000) which would self-assemble into an ordered lamellae. This physical approach to granule formation would only be possible if the amylopectin was synthesized to the proper structure. Thus, the enzymatic processes must be properly controlled so that amylopectin molecules can begin forming a granule. Studies involving mutants with altered functions of synthesis enzymes particularly SSIII and SSIV have revealed that when the enzymes are not present starch granule formation is altered (Roldán et al., 2007); Szydlowski et al., 2009). Amylose is not necessary for the formation of the starch granule as waxy mutants exhibit granules with similar physical properties as non-waxy starches.

The starch granule originates from the hilum (Ziegler et al, 2005) and alternating semi-crystalline and amorphous layers (lamellae) or growth rings are formed. The crystalline regions of the starch granule refract polarized light and produce a ‘Maltese cross’ phenomena which is characteristic of native ungelatinized starch (Buléon et al., 1998). A closer examination of the alternating lamellae by X-ray diffraction reveals that a 9-10 nm periodicity exists (Blanshard et al., 1984). This periodicity appears to be constant across all species which suggests a common method for starch deposition (Pérez and Bertoft, 2010). Further detail into the architecture of the starch granule can be found in the review by Pérez and Bertoft (2010)

The size and shape of the granules are affected by the species as well as the overall architecture of the cereal caryopsis. Starch granule size distribution has been measured by many techniques including, sieving (Evers et al., 1974), image analysis from light microscopy, and laser diffraction sizing (Wilson et al., 2006). Wheat, barley, rye, and oat typically express a bimodal or tri-modal starch granule size distribution (Bechtel et al., 1990; Parker, 1985; Wilson et al., 2006). The largest are referred to as A-type granules and are typically >15µm in diameter. The B-type granules range from 5-15µm in diameter and the C-type are <5µm in diameter. The A-type granules are lenticular in shape whereas the B- and C-type are commonly spherical in shape. The difference in granule size is thought to be the result of production in different phases of endosperm development (Parker, 1985). The small granules are possibly produced by amyloplast stromules (Langeveld et al, 2000; Bechtel and Wilson, 2003). The physiological purpose for the bi/trimodal distribution is unclear, but the small granules may be more efficient in carbon and energy storage (Tetlow, 2011). The starch granules in sorghum and maize exhibit a wide range of sizes and can also be polyhedral or spherical in shape (Tester et al., 2004; Benmoussa et al., 2006). Maize and sorghum starch granules also contain channels which penetrate the surface of the granule towards the hilum (Huber and BeMiller, 2000). Ultimately, the size controlling factor of the starch granules is not fully understood and further research is needed in this area.

The chemical composition of the starch granules consists primarily of a ratio of amylose and amylopectin, but small amounts of proteins and lipids are present. For many plants with a normal type starch the amylose is the minor component, comprising 15-30% by weight of the total starch content. Amylose is a mixture of linear chains of α-(1,4) linked D-glucose units. Some amylose molecules have α-(1,6) linkages occurring at approximately 0.3-0.5% of the total linkages. Since these branch points are usually separated by large distances the molecules tend to act essentially as linear molecules. The molecular weight distribution of amylose is reported to be in the 1.0×10^5 to 1×10^6 range or a DP range of approximately 800 to 3,000 (Buléon et al., 1998; Mua and Jackson, 1997). When amylose is present in an aqueous solution it is in a random coil arrangement with some single helical structures composed of six to eight glucose units per turn. Amylose will form complexes with other molecules that are seeking the hydrophobic environment of the interior of the amylose helix (Robyt, 1998).

The remaining 70-85% of the starch not comprised of amylose is made up as amylopectin. Amylopectin is a very large and highly branched polymer of D-glucose or even a polymer of amylose chains. Approximately 5% of the total linkages of amylopectin are the branch points or α-(1,6) linkages. The molecular weight distribution is in the 1.0×10^7 to 1×10^9 range making it one of the largest biological polymers (Buléon et al., 1998; Mua and Jackson, 1997). Amylopectin is present in all starches; however the typical ratio of amylose to amylopectin can be influenced by a few gene mutations. The most common mutation type that has been identified is the "waxy" starches. Waxy mutants exist in maize, barley, rice, wheat and sorghum and are identified by their lack of amylose. There has also been two maize varieties identified that are considered high amylose, with one variety being 50% amylose and the other 70% amylose. Some reports have indicated that there is a possibility of a 100% amylose maize starch (Robyt, 1998).

In addition to the amylose to amylopectin ratio another important characteristic of the starch is the fine structure of amylopectin. The branch chain-length distribution of the amylopectin is related to the crystalline structure of the starch (Hizukuri, 1985). The chain-length distribution can be found by debranching the starch with an enzyme, isoamylase, and separating the chains by either high-performance anion exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) or fluorophore assisted capillary electrophoresis using a laser induced fluorescence detector (FACE-LIF) (Hanashiro et al., 1996; Morell et al., 1998). The branch chain length distributions vary greatly depending on the botanical origin of the starch. Table 1.1 illustrates the differences. The A-type starches have a smaller average chain length than the B-type starches with the C-type falling in between the two (Jane et al. 1999). The very long chains of amylopectin allow for behaviors similar to amylose. These long chains are capable of forming helical structures with other components in a similar fashion to amylose chains.

Influences on Starch Synthesis and Functionality

Caryopsis Development

The development of the endosperm of cereal caryopses plays a key role in the synthesis, chemical and physical structures, and functional properties of starch. In wheat, it was observed that A-type granules were first detectable at around 3-4 days after anthesis (DAA) and new A-

type granules were developing through 14 DAA (Parker, 1985; Li et al., 2012). The smaller B-type granules form beginning around 12 DAA and the C-type granules appear around 22 DAA (Bechtel and Wilson, 2003). Starch granules are also detectable at 3 DAA in high-amylose rice (Qin et al., 2012). The starch component of the cereal grains undergoes a rapid increase in quantity between 12 and 35 DAA (Shewry, et al., 2009; Wang et al., 2014). This increase in starch content is associated with increases in the starch synthesis enzymes quantity which tended to peak around 15-18 DAA (Wang et al., 2014). The amylose content of the starch also increases through the development of the grain (Qin et al., 2012; Wang et al., 2014; Wei et al, 2010). As the amylose content increases a decrease in the crystallinity was also observed (Qin et al., 2012; Wei et al, 2010). The amylopectin structure is also altered due to the stage in maturity. In rice starches the molecular weight decreased as the grain advanced in maturity (Shu et al., 2014) and the amylopectin cluster degree of polymerization in wheat starch increased until around 28 DAA then decreased through maturity (Kalinga et al., 2014a). The average amylopectin chain length varied slightly due to kernel maturity but no clear trends were observed (Kalinga et al., 2014b).

The changes in the physical and chemical structures of cereal starches impact the functional properties such as gelatinization profiles, swelling power, and digestibility. The gelatinization temperature profiles were slightly altered at maturity, but the most noticeable effect was the decrease in the ΔH as the grain approached maturity (Qin et al., 2012; Wei et al, 2010). The swelling power of wheat starch appeared to decrease as the grain advanced in maturity (Wei et al., 2010). The resistant starch content of rice starch increased with the grain maturation (Shu et al., 2014).

Environmental Stresses

The agronomic traits of cereal grains are greatly affected by the environmental conditions. The environmental conditions also affect grain quality attributes. Heat and drought are the most common abiotic stresses in cereal grains (Campos et al., 2004; Prasad et al., 2006). Abiotic stresses can affect starch synthesis and the level and duration of the stress results in varying responses in the starch content and composition.

Drought or water stress is the most common stress and results in lower grain yield. Grain yield is well correlated with starch content, in barley starches were reduced up to 45% when water was withheld from 10 DAA until harvest (Worch et al., 2011). The starch synthesizing

enzyme activities are altered during drought stress, with SSs being the most sensitive. Not only is the overall starch content reduced, but also the composition, structure, and functionality are influenced by drought stress. The granule size distribution is altered by increasing the volume proportion of A-type granules (Fábián et al., 2011; He et al., 2012; Singh et al., 2008). The amylose content is lowered in both wheat (Singh et al., 2008), rice (Gunaratne et al., 2011), and triticale (He et al., 2012). The changes in granule size distribution and amylose content due to drought stress also affects the thermal properties of the starch, namely pasting and gelatinization. In rice flour peak viscosity, swelling power, cohesiveness, and gel hardness were increased after drought stress (Gunaratne et al., 2011). The gelatinization profile of drought stressed triticale was slightly altered exhibiting a narrower gelatinization range and an increase in enthalpy change (He et al., 2012). The effects of water stresses are variable due to the genetic tolerance for the drought stress.

Since there is a wide range of optimum temperatures for cereal grains responses to heat stresses are variable due to species. The responses for heat stresses were similar to drought stress, with a decrease in granule size raising the proportion of A-type granules, however the amylose response was variable. Amylose content decreased in maize (Lu et al., 1996), increased in wheat (Shi et al., 1994; Tester et al., 1996), and were unaffected in sorghum (Li et al., 2013). The gelatinization temperatures of cereal starches increase due to heat stress (Lanning et al., 2012; Matsuki et al., 2003; Lu et al, 2013).

Separation and Detection of Glucose by HPLC

The ultimate purpose of starch storage in cereal grains is to provide the energy required for the next generation of the plant to develop. However, since starch is such a great energy storage mechanism humans have adapted to utilize the stored energy, whether that be by consuming as food, feeding to animals, or producing fuel to power the mechanized world. Ultimately to unlock the potential energy stored in starch the structures synthesized must be broken back down to small sugars which can be used to power biological functions. In order to compare the starches on their functional properties the amount of sugars released must be quantified. Glucose and other sugars can be effectively separated and monitored using many analytical techniques.

One of the most useful tools in the separation and identification of chemical components of organic material is high performance liquid chromatography (HPLC). The principle behind HPLC is the analyte of interest is placed into a liquid mobile phase that carries it through a column packed with a stationary phase. Separation of the analyte is dependent on the type of chromatography being utilized. The separation of carbohydrates and specifically glucose can be achieved using multiple types of HPLC, including ion chromatography, reversed phase, hydrophilic interaction, and size exclusion. HPLC's versatility allows for the user to adjust the methods used to fit their chromatographic equipment.

Ion chromatography is perhaps the most commonly used form of HPLC to separate monosaccharides. There are two types of ion chromatography used: ion exchange and ion exclusion. Ion exchange chromatography works by binding the analyte to the stationary phase inside the column, utilizing the ionic interaction between oppositely charged molecules in a low concentration salt solution. Once the materials are bound a gradient of increasing salt concentration is used to begin separating the molecules. The weaker the ionic interaction the earlier the interaction will terminate and the analyte will elute from the column. The gradual changing of the mobile phase pH can also be utilized to separate the analyte from the stationary phase. Since carbohydrates are typically negatively charged, anion-exchange chromatography is employed as the separation mechanism. In anion-exchange the pH is lowered in a gradient; as the pH lowers the analyte becomes more protonated, and therefore less negatively charged, terminating the interaction. The functional group on the stationary phase is typically a quaternary amine or diethyleaminoethyl (DEAE). The sample elution time relatively long compared to other forms of chromatographic separation, but provides excellent resolution with high sensitivity. One disadvantage of ion exchange chromatography is that high pH ranges can cause epimerization or degradation of the sugars (Lee, 1990). Ion exchange chromatography has been reviewed in greater detail by Lee (1990, 1996).

Ion exclusion is the second type of ion chromatography that relies upon ion exchange columns to separate carbohydrates. The technique separates molecular species based on their ability to partition between the eluent and the eluent contained within the stationary phase resin. Cation-exchange columns are typically used with H+, Pb+2, Ca+2, or Na+ ions bonded to the stationary phase. Molecules with similar charges are repelled from the resin network, but neutral or oppositely charged molecules can enter the network and are eluted based on either their

interaction with the ions or on size exclusion (Tanaka and Fritz, 1987; Fritz, 1991). This chromatographic separation is typically done with an isocratic flow of a low concentration sulfuric acid or deionized water mobile phase. Ion exclusion chromatography provides good separation for beverages and foods that contain a large number of ionized compounds. Separation of glucose from other components can be done very quickly with good resolution. For example, glucose can be separated from other sugars and organic acids in fruit juices in under 15 minutes (Chinnici et al. 2005; Kelebek et al., 2009).

Reversed phase (RP) is the most widely used form of HPLC and has been used to separate monosaccharides (Shaw and Wilson, 1983; Dai et al., 2010; Zhang et al., 2013). This technique uses a stationary phase with hydrophobic functional groups attached with a polar mobile phase. The molecules of interest interact with the hydrophobic groups on the stationary phase and are eluted as the polarity of the mobile phase decreases. The more hydrophobic the molecule is the longer it remains in interaction with the column increasing its elution time. The mobile phase typically consists of an organic solvent, such as acetonitrile, the concentration of which is increased in a gradient throughout the sample run. When RP-HPLC is used to separate monosaccharides a C18 or C8 functional group is attached to the stationary phase. In carbohydrate analysis by RP-HPLC, the sugars are usually derivatized with an aromatic group rendering them hydrophobic. Due to the gradient nature of the separation RP-HPLC run times are longer than some other forms of chromatography, but recent advancements in column packing material has shortened the elution times (Bean et al., 2011).

Hydrophilic interaction chromatography (HILIC) utilizes stationary phases that are polar - similar to normal phase HPLC - but with mobile phases that are similar to those used in RP-HPLC. The most common stationary phase for sugar analysis is a silica particle with amino functional groups. The analytes are partitioned on the surface of the stationary phase and retained by hydrogen bonding. Samples are separated based on the number of polar groups and their conformation. The mobile phase can be a gradient of decreasing organic solvent concentration or an isocratic run of usually 70-80% acetonitrile. Glucose can be separated from other sugars in under 15 minutes in HILIC. A more extensive review of HILIC can be found by Jandera (2011).

Size exclusion chromatography (SEC) is commonly used to separate polymeric carbohydrates, but has been occasionally employed to separate glucose. SEC separates

molecules based on their hydrodynamic volume and requires molecular weight differences of 10-20% to achieve baseline separation. Either an aqueous or an organic solvent can be utilized as the mobile phase passing through a polymer or silica bead stationary phase. Typically SEC has relatively long run times and does have difficulty separating glucose from fructose (Giannoccaro et al., 2008)

The chromatographic separation is the first step in the analysis of glucose and other sugars. Once the glucose is separated it can be detected with several different types of detectors, including Refractive Index (RID), Pulsed Amperometric (PAD), Evaporative Light Scattering (ELSD), UV, and Fluorescence. The various detectors are not all suited for each type of chromatography used, therefore selection of a detector needs to match the separation process.

The RID is considered a universal detector since it is measures the difference in the refractive index of the sample eluent compared to the reference cell. RID will work with both aqueous and organic mobile phases. However, the measurement is sensitive to the liquid contained in the reference cell; any changes to the mobile phase creating differences to the reference cell liquid will cause baseline shifts or movement. The detector is also sensitive to changes in pressure and temperature. RID is very useful for sugar detection since native sugars do not contain a chromophore or fluorophore. Chromatographic separation techniques such as ion exclusion and size exclusion are commonly paired with RID since they operate with isocratic flow of the mobile phase. RID has been used to detect glucose in many food and industrial systems. Recently, Carballo et al. (2014) used ion exclusion with RID to separate the sugars of oranges and other citrus fruits, to quantify glucose they used a calibration curve with a range of 100-5000µg/L. A similar range was used with sugars in grapevine berries (0.1-20g/L) with an instrument detection limit of 0.16g/L (signal:noise =3) (Eyéghé-Bickong et al. 2012). Glucose was separated by HILIC with RID from milk powders with a level of detection at 29µg/mL (Ma et al., 2014). When paired with appropriate separation techniques, RID offers good detection on sample materials known to contain reasonably small amounts of glucose. RID does not offer the sensitivity of PAD or ELSD, but it requires less sample preparation than fluorescence and better detection than UV.

The most common detector system used with ion exchange chromatography is the PAD. Glucose and other carbohydrates are detected by PAD via the measurement of the electric current generated by their oxidation on a gold or platinum electrode. This oxidation at the

surface of the electrode creates a residue on the electrode which needs to be cleaned by increasing the volt potential which oxidizes the electrode and removes the carbohydrate oxidation product. Then the potential is lowered to reduce back to the original metal electrode. This utilization of three potentials creates the pulse or waveform which continuously repeats over the course of the sample analysis. PAD has been used to quantify glucose and other sugars in many food and bioindustrial systems including: honey (Ouchemoukh et al. 2010), chickpeas (Gangola et al., 2014), biomass from oranges (Widmer et al., 2011) corn stover (Wang et al., 2012), fruit juice (Zook and LaCourse, 1995) and debranched cereal starches (Yoo and Jane, 2002). PAD has become widely used due to its high sensitivity with a level of detection of 0.2μM for glucose (Zook and LaCourse, 1995, Gangola et al., 2014). Recent advancements in PAD for carbohydrate analysis has been extensively reviewed by Rohrer et al. (2013).

ELSD is considered to be a universal detection system and is commonly used to detect and quantify glucose as well as other carbohydrates. ELSD is a destructive detection technique that requires the nebulization and evaporation of the mobile phase leaving behind the analytes that are not evaporated. The analyte particles enter the detection region and scatter light onto a photomultiplier tube which measures the intensity as voltage. Since ELSD is compatible with many types of chromatographic separations used in sugar analysis it has been used to quantify glucose in many food systems ranging from milk, fruit and fruit juice, vegetables and cereal grains (Ma et al., 2014; Terol et al., 2012; Shanmugavelan et al., 2013). Bioindustrial applications such as biomass hydrolysis products have also been quantified with ELSD (Liu et al., 2012). ELSD offers a reported level of detection of 0.37μg/L (Liu et al., 2012) for glucose. A disadvantage to ELSD is that the calibration curve is not linear over large concentrations of analyte. The detector is also susceptible to mobile phase contaminants which can influence the signal:noise ratio and must be used with mobile phases that are volatile at the temperature range needed for analysis of the sample.

Fluorescence and UV light detection can be used to detect sugars but require more sample preparation steps or offer poor detection sensitivity compared to PAD or ELSD. Usually a derivatization process with a chromophores or fluorophores are needed for detection; however some studies have been done with UV detection at 190-195nm. The detectable threshold for glucose at that wavelength range was found to be 9μg/L (Shaw and Wilson, 1983). In order to achieve detection levels approaching or even exceeding PAD and ELSD the sugars need to be

tagged with either a UV absorbing chromophore, such as 1-phenyl-3-methyl-5-pyrazolone (PMP) or a fluorophore, such as 2-(12-benzo[b}acridin-5(12H)-yl)-acetohydrazide (BAAH). Dai et al. (2010) used RP-HPLC to separate tagged monosaccharides, with glucose having a detection limit of 0.13nmol. Fluorescence detection levels of 10µg/L were found after pre-column derivatization (Zhang et al., 2013). The detection of glucose by UV or fluorescence detection is not as popular as the other detection methods most likely due to the additional steps of tagging the sugars which can cause alterations to chemical structures as well as being subject to tagging efficiency.

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Table 1.1 Amylopectin Branch Chain Length Distributions from various starch sources

		% Distribution					Highest Detectable
Source	**Average CL**	**DP 6-9**	**DP 6-12**	**DP 13-24**	**DP 25-36**	**DP ≥ 37**	**DP**
A-type Starches							
Normal Maize	24.4	3.9	17.9	47.9	14.9	19.3	80
Waxy Maize	23.5	6.9	17.0	49.4	17.1	16.5	73
Normal Rice	22.7	4.1	19.0	52.2	12.3	16.5	80
Waxy Rice	18.8	8.6	27.4	53.4	12.6	6.6	66
Wheat	22.7	5.2	19.0	41.7	16.2	13.0	77
Barley	22.1	4.9	20.8	48.9	17.7	12.6	75
Tapioca	27.6	4.7	17.3	40.4	15.6	26.7	79
B-type Starches							
ae Waxy maize	29.5	2.3	10.4	43.5	18.1	28.0	84
Amylomaize V	28.9	1.9	9.7	43.9	20.3	26.1	86
Amylomaize VII	30.7	1.8	8.5	40.7	21.3	29.5	86
Potato	29.4	3.5	12.3	43.3	15.5	28.9	85
C-type Starches							
Lotus root	25.4	4.6	16.4	47.2	15.4	21.0	83
Green banana	26.4	5.3	16.8	46.3	12.9	24.0	79

Table adapted from Table III in Jane et al. (1999)

Chapter 2 - Development of a 96-well Plate Iodine Binding Assay for Amylose Content Determination.

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Abstract

Cereal starch amylose/amylopectin (AM/AP) is critical in functional properties for food and industrial applications. Conventional methods of AM/AP are time consuming and labor intensive making it difficult to screen the large sample sets necessary for evaluating breeding samples and investigating environmental impact on starch development. The objective was to adapt and optimize the iodine binding assay in a 96-well plate format for measurement at both λ620nm and λ510nm. The standard curve for amylose content was scaled to a 96-well plate format and demonstrated R^2 values of 0.999 and 0.993 for single and dual wavelengths, respectively. The plate methods were applicable over large ranges of amylose contents: high amylose maize starch at 61.7±2.3%, normal wheat starch at 29.0±0.74%, and a waxy maize starch at 1.2±0.9%. The method exhibited slightly greater amylose content values than the Concanavalin A method for normal type starches; but is consistent with cuvette scale iodine binding assays.

Introduction

Starch is synthesized and deposited in the endosperm of cereal grains to function as an energy reserve. In wild-type grains starch consists of two distinct polymers, amylose and amylopectin. Amylopectin is a large, highly branched polymer consisting of α-1,4 linked D-glucose units with branches linked by α-1,6 bonds. Amylose is a mostly linear polymer of α-1,4 linked D-glucose with a few α-1,6 branch points. In wild-type starches amylose content is usually in the 20-30% range, however mutants exist for several cereals that contain very high (>40%) and very low (0-15%) levels of amylose (Tester, Karkalas, & Qi, 2004).

The ratio of amylose to amylopectin is important to both the functionality and the nutritional properties of starch and starch based products. Amylose is important to the thermal characteristics of starch, such as gelatinization and pasting (Jane et al., 1999, Sasaki, Yasui, & Matsuki, 2000). The ratio of amylose:amylopectin also influences starch retrogradation, a major issue in the staling of food products (Hug-Iten, Escher, & Conde-Petit, 2003). Foods with a high amylose content have been shown to have a reduction in glycemic impact, which promotes many health benefits such as better control of diabetes and obesity (Behall & Scholfield, 2005).

There are currently several methods utilized for amylose content determination, ranging from high-performance size exclusion chromatographic techniques (Batey & Curtin, 1996; Chen & Bergman, 2007; Kennedy, Rivera, Lloyd, & Warner, 1992) to differential scanning calorimetry (Mestres, Matencio, Pons, Yajid, & Fliedel, 1996). The most commonly used methods are based on binding of either amylopectin or amylose with another compound. The method in which amylopectin is precipitated with Concanavalin A, developed by Yun and Matheson (1990) and modified by Gibson, Solah, & McCleary (1997), has recently increased in use due to advantages it possesses over other methods. The method can be commercially purchased as a kit and does not require a standard curve to quantify amylose. While effective, all the above methods are very labor intensive, time consuming and not conducive to screening large numbers of samples, such as is needed for evaluating breeders' samples.

Another widely used method has been the measurement of iodine binding of amylose producing a blue coloration. The iodine-binding method was introduced by McCready and Hassid (1943) for measurement of amylose in potato starch. Since the introduction many modifications have been made to the procedure, adjusting for sample preparation, standards, and measurement wavelength (Juliano, 1971; Juliano et al., 1981; Knutson, 1986). The amylose

content was commonly overestimated due to interference from the amylopectin-iodine complex, possibly due to the complexation of iodine and the amylopectin side chains. Recently, Zhu, Jackson, Wehling, & Geera (2008) evaluated many amylose content measurement techniques and developed a method utilizing a dual-wavelength approach. The dual-wavelength method had greater precision and accuracy than the single wavelength method due to a reduction in the effect of the amylopectin-iodine complex. Many of the amylose content measurements are capable of providing accurate and precise measurements; however the greatest disadvantage to all of the methods is the speed of measurement or the number of samples that can be analyzed in a day.

The measurement of quality traits in breeding populations of cereal grains is becoming more important. Plant breeders evaluate the end-product quality of their breeding lines at very early stages in the process, when populations number in the hundreds to thousands. Current amylose content measurements are very time consuming and low throughput, thus screening breeders' populations is very difficult and not commonly achieved. Therefore, the objectives of this study were to (1) develop a method capable of analyzing 50-100 samples of starch per day and (2) maintain a level of precision and accuracy needed for screening.

2. Materials and Methods

2.1 Materials

Starches from waxy maize, high amylose maize, normal maize, high amylose barley, and rice were commercially produced. Sorghum hybrids and wheat varieties were laboratory scale milled into flour and starch was isolated by the sonication method of Park, Bean, Wilson, & Schober (2006). All chemicals used were reagent grade. Amylose from potato (product number 10130, Fluka, Sigma Aldrich) and amylopectin from maize (product number 10120, Fluka, Sigma-Aldrich) were used as controls for preparation of the standard curves.

2.2 Conventional Amylose Measurement

Amylose content was measured on starch from all samples in replicate using the Concanavalin A precipitation method (K-AMYL, Megazyme International, Wicklow, Ireland).

2.3 Development of 96-well Plate Method

The method reported here modified the starch suspension methodology used by Hogg et al. (2013) and combined with the analysis wavelengths reported by Zhu et al. (2008). First, 5mg of starch sample or standards were weighed into 2mL centrifuge tube. Next 1ml of 90% DMSO in water was added and tubes heated to 95°C for 60 minutes with vortexing every 10 minutes. After starch dispersion, samples are cooled for 5 minutes and 100µL from each sample tube was added to a well on a 96-well plate. The standard curve for amylose content was prepared using both amylose and amylopectin, the ratios can be found in Table 2.1. After the samples were placed into the 96-well plates, 100µL of 90% DMSO with 3.04g/L iodine (due to I_2's solubility in DMSO the traditional I_2:KI solution was not necessary) was added to each well and plate was shaken for 2 minutes. The control blank, 100µL of 90% DMSO plus 100µL of 90% DMSO with 3.04g/L iodine, was placed into duplicate wells. A subsample (20µL) from each well was removed using a 96-well pipetting system and added to an empty plate, then180µL of deionized water was added to each well using the pipetting system and plate was shaken for 2 minutes. After agitation the 96-well plate was analyzed for absorbance at 620nm and 510nm. The absorbance was blanked with the control for a final ABS620 and ABS510 reading. A flow chart of the method can be found in Figure 2.1.

2.4 Amylose Content Calculation

A regression equation was determined for the standard curve on each plate analyzed using both the absorbance value at 620nm and the Diff ABS (ABS620-ABS510). The amylose content of the samples was calculated using these equations. Single wavelength amylose = (ABS620- y-intercept of regression / slope of regression); Dual wavelength amylose = (Diff ABS - y-intercept of regression / slope of regression).

2.5 Statistical Analysis

All analyses were conducted in quadruplicate unless otherwise stated. The means, standard deviation, and coefficient of variation were calculated using an Excel spreadsheet (Microsoft Corp., Redmond, WA). The coefficient of determination for the standard curve was found using OriginPro8 software (OriginLab Corp., Northhampton, MA)

3. Results and Discussion

3.1 Standard Curve

Initial testing to produce a standard curve utilized only amylose as a standard. It was found that a combination of purified amylose and purified amylopectin produced a better standard curve, which was similar to many previous studies. The plate method was capable of producing a highly accurate standard curve with both the single (ABS620) and the dual wavelength approach. Figure 2.2. shows a standard curve for both the single and dual wavelength methodologies. Since there may be slight variations in the plates, a standard curve was generated with every plate analyzed. The regression equation on the single wavelength assay had an R^2 of 0.999 on many plates and the values for the slope and y-intercept were very repeatable. The dual wavelength method also exhibited a very high coefficient of determination (R^2 =0.993) with plate to plate repeatability on slope and intercept values. The coefficients of determination were very similar to values reported for standard curves produced in larger cuvette scale methods (McGrance, Cornell, & Rix, 1998; Zhu et al, 2008). The level of accuracy and repeatability of the standard curves allows for quantification of amylose in starch samples.

3.2 Measurement of Amylose in Cereal Starches

The amylose content of the cereal starches analyzed can be found in Table 2.2. The values found for amylose by the Concanavalin A method and the 96-well plate methods developed all were within the range normally expected for the respective sample. The singe wavelength (ABS 620) prediction of amylose content tended to be slightly higher than the Concanavalin A values. The overestimation of amylose on a single wavelength iodine binding had been previously reported by Zhu et al. (2008). Recently, a study showed that increases in amylopectin chain length lead to a greater disparity between the iodine binding amylose content and Concanavalin A values (Park, Kim, Chung, & Shoemaker, 2013), suggesting that amylopectin interferes with the single wavelength detection.

The dual wavelength detection of the iodine binding used in this methodology appears to reduce the overestimation and improve the precision and accuracy when compared to the single wavelength approach. The dual wavelength method produced values for amylose content very similar to the Concanavalin A measurement. The 96-well plate dual wavelength method is capable of analyzing amylose in waxy to high-amylose types of starch (Table 2.2). Previous

attempts at high-throughput plate methods experienced difficulty achieving similar Concanavalin A values and iodine binding values for normal starches (Hu, Burton, & Yang, 2010) or produced values with high coefficients of variation (Hogg et al., 2013). These variations may be due to difficulties with interference due to amylopectin-iodine interactions or standard curve preparation techniques.

The dual wavelength method's precision was tested on wheat starch with 10 replicate samples. The mean amylose content of the starch was found to be 29.0% with a standard deviation of 0.74%. The coefficient of variation for these 10 replicates was 2.55%, which is slightly lower than the common 5% value of the Concanavalin A measurement. The coefficient of variations found with four replicates of the other starches showed a range from 2.44% to 4.14% in normal and high amylose starches. The waxy maize starch had very large coefficient of variation due to mean values being close to zero. This suggests that there may be some difficulty in statistical separation of waxy starches from other waxy starches; however this is not a concern for normal and high-amylose starches.

Normal and high-amylose starches contain lipids which can form complexes with amylose and affect the intensity of the blue color formed by the amylose-iodine complex (Morrison and Laignelet, 1983). Lipids in those cereal starches need to be removed to obtain absolute amylose content, thus amylose measurements in this study are apparent amylose values.

4. Conclusions

The adaptation of several iodine binding methodologies allowed for an accurate and precise amylose content determination. The 96-well plate method is capable of analyzing over 50 samples in replicates on a daily basis. This speed of analysis is a drastic improvement over current methodologies and will allow for timely screening of large breeder populations. Since the accuracy of amylose measurement is not effected by the speed of measurement this method could become an effective tool for early generation quality testing using relatively small quantities of isolated starch.

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Table 2.1. Standard Curve Preparation.

Amylose Content %	Amount of 5mg/mL Amylose Solution (µL)	Amount of 5mg/mL Amylopectin Solution (µL)
0	0	100
5	5	95
10	10	90
15	15	85
20	20	80
25	25	75
30	30	70
50	50	50
75	75	25
100	100	0

Table 2.2 Comparison of amylose content estimates for cereal starches.

Cereal Starch	Amylose Content (%)		
	Concanavalin A Assay	Single λ 96-well Plate	Dual λ 96-well Plate
Rice	18.5±0.88	21.5±1.16	19.6±0.54
High Amylose Barley	36.5±0.05	42.9±1.99	39.4±0.96
HYLON V Maize	49.7±3.06	68.1±3.43	49.2±1.45
Wheat	28.9±0.28	30.9±0.65	29.0±0.74
Sorghum	29.1±0.92	28.1±1.34	30.4±1.26
High Amylose Maize	59.5±0.31	78.5±1.15	61.7±2.30
Waxy Maize	2.4±0.70	0.24±0.73	1.2±0.90

Figure 2.1 Flow Chart for 96-well Plate method.

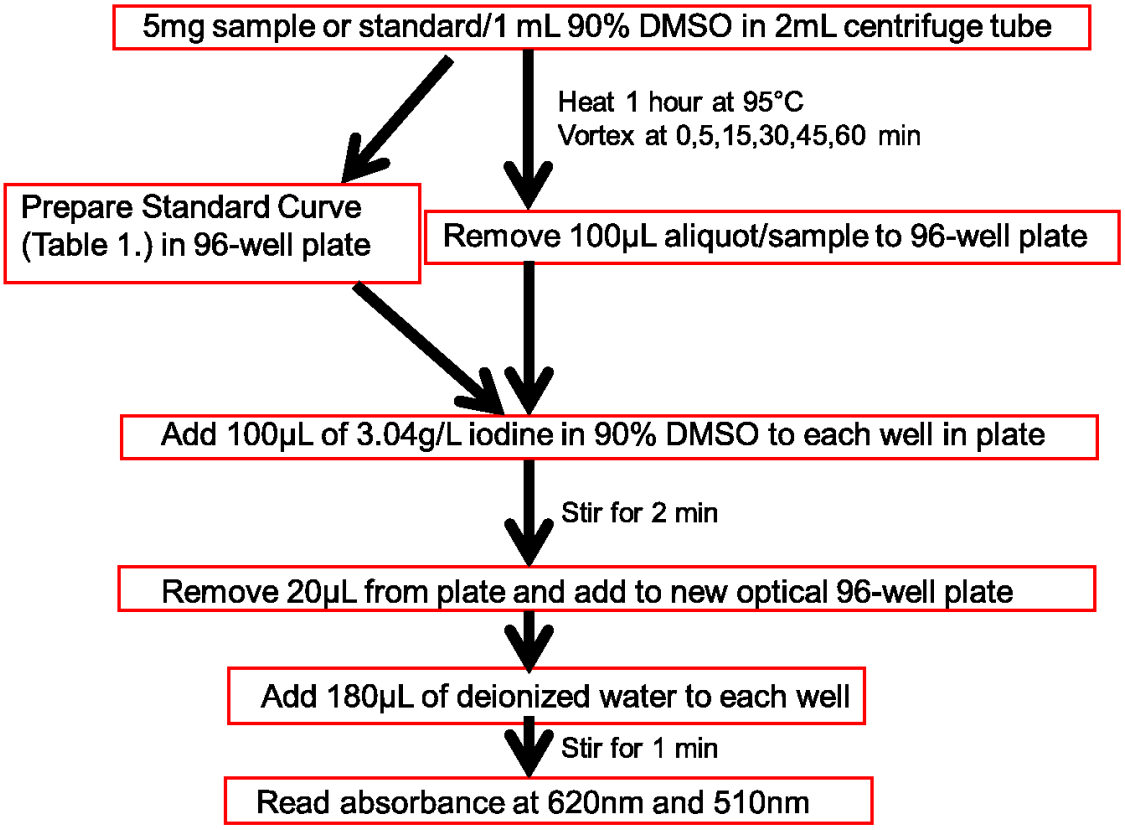
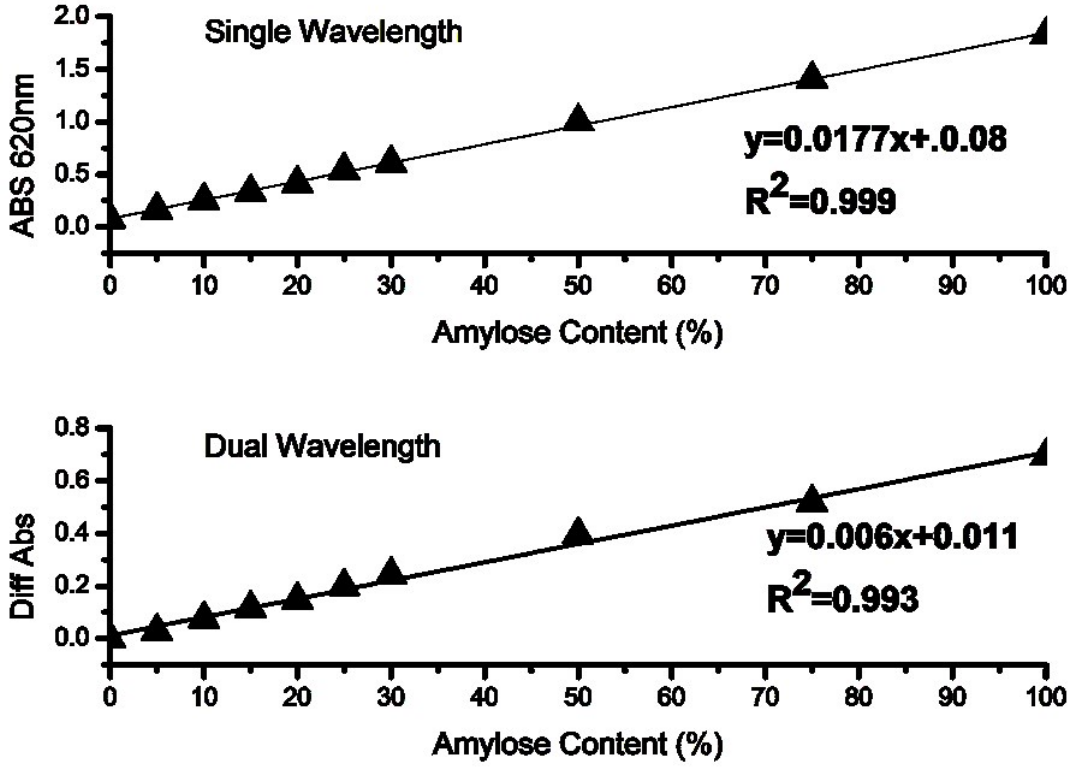


Figure 2.2 Regression equations for amylose standard curve prepared in 96-well plate.



Chapter 3 - Sorghum starch properties as affected by growing season, hybrid, and kernel maturity

ABSTRACT

Starch is a widely used component in the food, feed, and biofuel industries. A critical component in the functionality of a starch in a food or industrial system is the thermal properties of the starch. The objective of this study was to examine the development of the starch granule through kernel development and determine if sorghum kernel development impacts the properties of starch. Two sorghum hybrids were grown in an irrigated plot in 2008 and 2009; upon reaching the mid-bloom stage in maturity approximately 200 heads were tagged in each plot. Samples were collected beginning ten days after anthesis (DAA) until harvest. The samples were then decorticated and the starch was isolated. The starch granule size distribution was greatly affected by the collection date as well as the growing season and hybrid. The samples ranged from 16.3% amylose in 10 DAA to 23.3% amylose in 35 DAA. The crystallinity of the starch decreased as the DAA approached physiological maturity (35 DAA). Starch thermal properties were also altered due to DAA, most notably the ΔH was 16.1 J/g at 14 DAA, 11.95 J/g at 35 DAA, and 9.45 J/g at 56 DAA. The unique chemical and thermal properties of the starches could allow for utilization of the starch in differing applications.

INTRODUCTION

Sorghum is fifth in worldwide cereal grain production; behind maize, rice, wheat, and barley. However, in the United States sorghum production ranks third behind only maize and wheat (FAOStat, 2014). Sorghum is used in many different applications, such as animal feed, biofuel feedstock, and increasingly in food systems. Compositionally sorghum is very similar to the other cereals; however both the starch and protein are less digestible than the other cereals (Hamaker et al. 1986, Rooney and Plugfelder 1986). The end-product functionality of sorghum is directly related to its starch chemistry and structure.

The characterization of starch chemical properties is done on multiple levels of starch organization. Perhaps the most basic is to compare the ratios of amylose to amylopectin found in the starches. Differing amylose content values can lead to changes in the thermal properties of the starch (Jane et al. 1999). The molecular organization of the amylopectin is also important to the functionality of starch. Molecular weight distributions and side chain distributions can be effected by genetic and growth condition factors. Variance in these distributions can also affect the functional properties of the starch (Jane et al. 1999; Fredriksson et al. 1998). The chemical composition of the starch can influence the granular structure of the starch (Jenkins and Donald 1995).

The amylopectin and amylose are packed into discreet bundles called granules (Zobel 1988) which are the highest level of starch structure. The physical properties such as size and shape of the starch granules can affect the functionality of the starch in many applications. Starch granule size distribution has been shown to influence thermal properties as well as digestibility (Eliasson and Karlsson 1983 and Chiotelli and Le Meste 2002). The shape of the granules can also influence its functional properties; Benmoussa et al. (2006) found that sorghums that had “doughnut-shaped” starch granules were more digestible. The development of the kernel affects the both the structure and chemical components of the cereal grain.

The ultrastructure of wheat endosperm during kernel development was studied by Bechtel and Wilson (1997) to relate starch granules and storage proteins to hardness. In sorghum a study was conducted by Van Scoyoc et al. (1988) to examine the changes in kernel characteristics as well as endosperm protein fractions as kernels developed. However, there has been little research into the development of starch granules or the investigation into the starch properties of developing sorghum.

The objective of this study was to 1) investigate the development of the sorghum starch granule during kernel development 2) investigate the effect of maturity on the starch chemistry and thermal properties.

MATERIALS AND METHODS

Sample Collection and Processing

The sorghum hybrids "Seneca" and "TX631*TX436" were grown at the Kansas State University Ashland Bottoms Research Farm during the growing seasons of 2008 and 2009. Weather data was collected by the Kansas State University weather data library for the two growing seasons. Seneca is a medium-early (64 days to relative maturity) maturing hybrid with a purple plant color and grain that has a bronze pericarp with a hetero-yellow endosperm. TX631*TX436 is a late maturing (76 days to relative maturity) hybrid that has a white pericarp and endosperm grain on a tan plant. Approximately 200 panicles of sorghum were tagged at the mid-bloom stage (when half of the panicle's florets were flowering), with the date of tagging was considered the day of anthesis. Then five panicles were harvested on eleven collection dates, ranging from 10-56 days after anthesis (DAA). All sorghum samples were threshed and cleaned (mechanically or by hand dependent on the stage in development), then decorticated using a tangential abrasive dehulling device (Venables Machine Works, Saskatoon, SK, Canada) or hand peeling of the pericarp before starch analysis. The samples were typically suitable for mechanical cleaning and decortication at around 21DAA. Decorticated sorghum was ground using an Udy mill (Udy Corp., Fort Collins, CO) with a 0.5mm screen.

Grain Imaging

The cleaned grain from all collection dates for the sorghum hybrid Seneca grown in 2008 were imaged using a Keyence VHX-1000 digital microscope. The samples were imaged both whole and cross-sectioned. Images were analyzed using ImageJ software.

Starch Isolation

Starch was isolated from the decorticated sorghum meal by the sonication method of Park et al. (2006). Five grams of meal was mixed with 100mL of a 12.5mM sodium borate buffer, pH 10, with 0.5% sodium dodecyl sulfate (SDS) and 0.5% sodium metabisulfite. The mixture was

then sonicated for 100 seconds with a sonication amplitude of 75% using a VCF-1500 ultrasonic processor (Sonic & Materials, Newtown, CT) equipped with a 25.4mm probe. Sonication was conducted in a glass jar placed in ice water to prevent heat buildup in the sample. After sonication the samples were centrifuged (2000 x g, 10 minutes) and the supernatant was decanted. The starch pellet was resuspended in approximately 80 mL of deionized water and the resulting slurry was passed through a 62µm screen to remove any residual bran. The slurry that passed through was then centrifuged (2000 x g, 5 minutes) and the supernatant was decanted. The starch pellet was washed two additional times in 40mL of deionized water, and then freeze dried. The dried starch was ground with a coffee grinder (Model IDS 55, Mr. Coffee, Boca Raton, FL) and then used for subsequent starch physical and chemical analyses.

Starch Granule Size

Starch granule size distributions were measured using a single wavelength Beckman Coulter LS 13 320 Particle Size Analyzer (Miami, FL) with the Universal Liquid Module (ULM) for liquid-based measurements. Since a visualization of shape is not possible with laser diffraction sizing the granular structure of the starch in these samples will be defined by their size and volume.

Amylose Content

Amylose content was measured using a dual-wavelength iodine binding method conducted in 96-well microplates (Kaufman et al., 2015).

X-Ray Scattering

A subset of the starch samples were analyzed by small-angle X-ray scattering (SAXS) and wide-angle X-ray cattering (WAXS) at the X27C beamline athe the National Synchrotron Light Source (Brookhaven National Laboratory). The subset of samples consisted of five collection dates from 2008 on both Seneca and TX631*TX436. The collection dates were 10 DAA (earliest in development), 17 DAA (midpoint between anthesis and physiological maturity), 28 DAA (near physiological maturity), 35 DAA (beginning of kernel drydown), and 56 DAA (typical harvestable moisture content). Details of the experimental setup at the X27C beamline have been reported by many others (Chen et al., 2006; Chu and Hsiao, 2001, Cai et al., 2010). WAXS was conducted on both dry starch and hydrated starch (~50%). A 2D MAR-CCD

X-ray detector was used for data collection and Polar software (Precision Works NY, Inc.) was used for data processing. The percent crystallinity of the starches was calculated by the ratio of the total crystalline peak diffraction intensity to the total diffraction intensity.

Amylopectin Branch Chain Distribution

Isolated starch was suspended in 90% DMSO and heated at 95°C for 60 minutes. A 100µL aliquot was removed to a 1.5mL centrifuge tube and 300µL of 95% ethanol was added with vortexing to precipitate the starch. The tube was then centrifuged (6800 x g, 15 minutes) and supernatant removed. A 500mM sodium acetate trihydrate buffer (pH 4.4) (250µL) was added with vortexing and 10µL of isoamylase solution (250U/mL) was then added. Tubes were mixed and allowed to incubate for 16 hours at 42°C. After debranching with isoamylase 10µL of digesta was vacuum dried on a SpeedVac (SC110, Savant Instruments, Inc., Holbrook, NY) for 4 hours. The reducing ends of the debranched starch in the dried pellet were derivatized with the 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) by suspending samples in 2 µL 1M sodium cyanoborohydride in tetrahydrafuran and 2 µL APTS (10mg/96µL in 15% acetic acid) and incubating overnight at 42 °C. After tagging, 46µL of deionized water was added and tube was mixed and centrifuged (13,000 x g, 2 minutes). 10µL of the supernatant was removed and added to 190µL of deionized water and sample was injected (20 sec at 5 psi) onto a Beckman MDQ (Beckman Coulter, Inc., Brea, CA) capillary electrophoresis instrument. Separation was achieved using a 61.2cm silica capillary (50cm to detector) with a voltage separation at 23.7kV in reverse polarity for 50 minutes. The N-Linked Carbohydrate Separation Gel buffer solution (Ref #477623, Beckman Coulter, Inc., Brea, CA) was used as the run buffer, with peak migration monitored using laser induced fluorescence at excitation wavelength of 488nm. Prior to sample injection the capillary was rinsed first with 0.1M NaOH (5 minutes at 40 psi) then with run buffer (3 minutes at 20 psi). Corrected peak area calculations were determined using 32 Karat software.

Molecular Weight Determination

Isolated starch samples were weighed (5mg) into a 2mL centrifuge tube and 1mL of 90% DMSO was added and tube was heated for 60 min at 95 °C with vortexing every 10 minutes. After heating a 200µL aliquot was removed and starch was precipitated with 1.5mL of 95% ethanol, samples were centrifuged at 6800 g for 15 minutes. The supernatant was removed and

samples were resuspended in 1mL of boiling deionized water and heated at 100 °C for 30 minutes. Samples were then filtered with a 5.0μm nylon syringe filter before injection on HPLC. Hydrodynamic chromatography (HDC) methodology as described by Rolland-Sabaté et al. (2011) was used for separation and analysis of filtered starch samples. An Agilent 1200 HPLC (Agilent Technologies, Torrance CA) equipped with a Shodex KW-802.5 column and a KW-G guard column (Showa Denko Tokyo, Japan) was used for separation. The columns were maintained at 30 °C and deionized water at 0.5mL/min was used as an isocratic mobile phase. The elution was monitored with multi-angle light scattering (MALS) detection with refractive index as the concentration detector. The MALS detector (Dawn Heleos, Wyatt Technology, Santa Barbara, CA) was calibrated with toluene and normalized using a 30,000 Mw dextran. The specific refractive index increment (*dn/dc)* of 0.147 was used for molecular weight calculations. Astra 6 software was used to make weight average molecular mass (M_w) and radius of gyration (R_g) calculations.

Thermal Properties

The thermal profiles of the developmental sorghum starches were measured by differential scanning calorimetry (DSC) (Diamond Differential Scanning Calorimeter, PerkinElmer, Waltham, MA). Starch was suspended in water (1:3) in stainless steel pans, thermal profile was analyzed from 5°C to 130°C at a 10°C/minute rate. The samples were retrograded for one week at 4°C and rescanned using the same protocol. Pyris software was used to analyze the thermograms to determine gelatinization temperatures and enthalpy changes.

Statistics

The study was conducted with a completely randomized design and samples were analyzed in duplicate. Statistical software (SAS v 9.4, SAS Institute, Cary, NC) was used to make Least Significant Difference (LSD) determinations. PROC CORR was used for correlation analysis among the starch properties.

RESULTS

Weather conditions during growing seasons

A brief summary of the weather conditions during the growing seasons can be found in Table 3.1. The conditions were measured from planting until 56 DAA for each respective hybrid. Generally speaking 2008 was a warmer growth season with temperature averages nearly 2°C higher for both maximum and minimum temperatures. There was also approximately 1.7 times the precipitation in 2008 than that of 2009. Since the temperature were warmer in 2008 there were more growing degree days accumulated during the growth season, especially during grain fill, which led to a more rapid maturation.

Grain Morphology

Images were taken of the whole kernel as well as longitudinal and latitudinal bisections of Seneca across the collection DAAs (Figure 3.1). Naturally the smallest kernels occur earliest in development, the 10 DAA sample was 3mm in diameter. In the cross section images the 10 DAA appears to be filled with an opaque milky endosperm that lacks any substantial structure or order. As the grain approaches physiological maturity (28 DAA), the kernels were approximately 4mm in diameter. The black layer is present in the cross sections of the kernels and the differentiation of the vitreous and floury endosperm is beginning to appear. The germ or embryo is also clearly defined at this stage in development. The pericarp is also beginning to turn to its final mature color. When the grain is at a typical machine harvestable maturity (56 DAA) the pericarp has reached its final coloration and is 4mm in diameter. In the cross section images the two types of endosperm are clearly defined.

Starch Isolation

The starch yield from isolation ranged from 49%-78% of sorghum meal (dry basis). The starch yield was variable due to the low total starch content found in the early maturity stages. The starch ranged from 0.23-0.54% protein and 0.18-0.30% ash.

Starch Granule Size Distribution

Starch is bundled into packets referred to as granules; therefore its functional properties are dependent on both the physical properties of the granules and the chemical makeup of the

starch. In wheat, starch granule size distribution volume percent was binned into the classical three groups: A-type granules (>15µm), B-type granules (5-15µm), and C-type granules (<5µm). Since the classical groupings are vague and commonly associated with wheat starch not sorghum starch, a further breakdown of size ranges was also conducted. This set of groupings divides the granules distribution into six groups: <2µm, 2-5µm, 5-10µm, 10-20µm, 20-30µm, and >30µm. Groupings based on wheat starch types and the six size groupings were both calculated for comparison. The starch granule size distribution showed statistically significant variation for the growing season, hybrid, and DAA.

The effect of the growing season on the starch granule size distribution can be seen in Table 3.2. Samples across both hybrids and all DAAs grown in 2008 had a greater percentage of A-type granules than samples grown in 2009 (38.83% to 31.64%). This difference was more clearly displayed in the size grouping of 20-30µm, where volume percent was 14.59% in 2008 and 9.16% in 2009. Conversely samples from 2009 exhibited greater volume percentages of B-type and C-type granules. The <2µm, 2-5µm, and 5-10µm groups all were significantly different for growing season. However, the 10-20µm grouping showed no difference. This grouping contains the largest volume percentage of the groupings.

Since the sorghum hybrids in this study differed in maturity, a variation in grain fill period was expected. This variation in grain fill duration may have contributed to the differences seen in the starch granule size distributions of the two hybrids across both growing seasons and DAAs (Table 3.3). Seneca, an early maturing hybrid, had a greater percentage of A-type granules than the Tx631*Tx436 hybrid. Seneca had significantly greater volume percentages of granules in 10-20µm, 20-30µm, and >30µm groupings than the Tx631*Tx436 hybrid. The Tx631*Tx436's higher volume percentage in the <2µm, 2-5µm, and 5-10µm groupings suggest that it continues granule initiation longer than Seneca or is slower to add to the existing larger granules.

The development of the kernel showed a large shift in the size distribution of the starch granules. Figure 3.2 shows this shift in a sample that is early in development (10 DAA), a sample that is near physiological maturity (28 DAA), and a sample that is near harvest (56 DAA). In the 10 DAA sample the starch has a tri-modal distribution, but as the kernel matures the starch granule distribution shifts to a bi-modal distribution. Significant differences were observed in all three groupings (Table 3.4). For example, the 10 DAA sample's volume percent

breakdown was 4.19% A-type granules, 54.17% B-type granules, and 41.64% C-type granules compared to the 56 DAA samples that had a distribution of 55.6%, 34.23%, and 10.17% respectively.

Amylose Content

Starch is a unique system in that it is comprised of two types of polymers, amylose and amylopectin. The ratio between the two components is an important factor when considering the functional properties of the starch. Samples grown in 2008 were slightly higher in amylose content than those grown in 2009 (Table 3.2). The two hybrids grown for this study were both normal type starches and did not significantly differ in amylose content. The amylose content in the developing samples showed a trend of increasing as the starch granules matured then maintain their level through kernel dry-down (Table 3.4). The samples ranged from 16.3% amylose in 10 DAA to 23.3% amylose in 35 DAA.

Wide-Angle X-Ray Scattering

The starches across both hybrids and all maturity dates exhibited A-type WAXS patterns (Figure 3.3),however the Bragg angle values for the peaks are slightly different than traditional WAXS patterns due to the wavelength of the X-ray source (Cai et al, 2012; Bai et al, 2014). The samples earlier in development appear to have a slightly greater intensity, perhaps most notably the 16 degree 2Θ peak. The crystallinity of the hydrated samples was highest at 10 DAA at 15.1% in Seneca and 16.6% in Tx631*Tx436 (Table 3.5). As the Tx631*Tx436 samples progressed in development the crystallinity decreased until physiological maturity and remained steady through kernel dry down. The Seneca hybrid followed a similar trend however the 56 DAA had a slightly greater percent crystallinity than 35 DAA.

Small-Angle X-ray Scattering

The SAXS peak located at 0.6-0.65 q nm^{-1} appears to become broader and less defined as the kernel advanced in maturity. The peak was largest at 10 DAA for both hybrids. This suggests that the lamellar period is reduced as the starch progresses in development.

Amylopectin Branch Chain Distribution

The amylopectin molecule was debranched revealing the relative distribution of the side chains. An example electropherogram can be seen in Figure 3.5. The corrected peak area % was found for each peak and the peaks were grouped according to Hanashiro et al. (1996). There were no statistical separations due to year, hybrid, or DAA (Tables 3.2, 3.3, 3.4). The distributions were approximately 28.5% in DP 6-12, 54% in DP13-24, 12% in DP25-36, and 5.5% DP>37. The highest detectable DP was 70.

Molecular Weight Determination

The amylopectin weight average molar mass (M_w) showed significant differences for both the growing season and hybrid (Tables 3.2 and 3.3). The samples grown in 2009 had an M_w of 9.6×10^7 versus an M_w of 8.6×10^7 in 2008. The hybrid Seneca had an M_w of 10.8×10^7 across both growing seasons with Tx631*Tx436 having a much smaller molar mass. In the ANOVA a significant interaction ($p<0.0001$) of year*hybrid was observed suggesting that the M_w is variable to this interaction as well as the individual factors. There was also significant interaction between the year*DAA ($p=0.0065$) and strong interaction between year*hybrid ($p=0.0562$). These interactions confounded the variation due to DAA which was not significant and lead to no mean separations for DAA (Table 3.4).

The root mean square radius or radius of gyration (R_g) was also calculated with the MALS data. There were significant differences in the R_g values due to both the growing season and the hybrid (Tables 3.2 and 3.3). The R_g values were not significantly affected by the DAA, but were significant for all forms of interactions between the year, hybrid, and DAA.

Thermal Properties

The gelatinization of starch is important component of functionality in food, feed, and industrial applications. Gelatinization range was measured with onset, peak, and end temperatures as well as the change in enthalpy (ΔH) or energy required to gelatinize. There were significant differences in all gelatinization measurements for growing season. Samples required slightly more energy to gelatinize and higher temperature ranges were seen in the 2008 growing season (Table 3.6). The hybrid Seneca gelatinized at a higher temperature range than Tx631*Tx436, however a greater ΔH was observed in Tx631*Tx436.

The kernel maturity showed a large effect on the gelatinization profiles. The gelatinization onset temperature was higher in sample leading up to physiological maturity. There were no significant differences in samples up to 31 DAA. The 35 DAA thru 56 DAA samples comprised a second statistical grouping which was not separated from each other, but lower than the early maturity samples. The peak and end gelatinization temperatures were similar to each other across all maturity dates with only slight separations, but no clear trends were observed. Perhaps the most interesting finding was in the ΔH which was highest at 14 DAA and began to decrease as the samples approach physiological maturity. The ΔH of 28-35 DAA samples were significantly different than the early maturity and the dried down samples. The ΔH was lowest in the samples during kernel dry down.

Over time under proper conditions the gelatinized starch will partially recrystallize or retrograde. When the starch samples were reanalyzed in the same manner as for gelatinization profiles the retrograded starch will melt or regelatinize. This melting occurs at lower temperatures due to the prior disruption of the granules. The degree of retrogradation can be measured by this formula: ΔH of retrograded sample/ΔH of gelatinization. There was little difference seen in the retrogradation temperatures for both the hybrid and growing season (Table 3.5). The samples grown in 2008 exhibited a greater degree of retrogradation at 26.9% compared to 23.1% in 2009. A hybrid effect was also seen for the degree of retrogradation, Tx631*Tx436 at 26.3% to Seneca at 23.7%. Samples early in maturity had an onset temperature lower than samples at maturity and drying down, but little separation was seen at the peak or end temperatures. The ΔH's were higher in the early stages of development and decreased as the sample reached maturity. Since the trend was the same as in gelatinization, there were not any clear trends in the degree of retrogradation due to the kernel maturity.

DISCUSSION

This study closely examined the roles of genetics, environment, and kernel maturity on the starch physical and chemical attributes. Even though, the samples were grown in the same location for the two years with the same cultural practices the weather conditions provided enough differences to create different environmental conditions. These differences in the genetic makeup of the sorghums tested as well as the environmental differences lead to many statistical differences in the starch properties.

The warmer and wetter conditions in 2008 allowed for starch granules to grow larger than in 2009. The conditions also lead to an increase in the overall amylose content of the samples. Previous work on heat stress in sorghum has shown that granule size decreased but amylose content was unaffected by increased temperatures (Li et al., 2013), however this was a controlled heat stress study with temperatures much greater than reported for the 2008 growing season. The amylose content of wheat starch increased with increasing temperatures after anthesis (Shi et al., 1994) Water stresses in cereal grain development typically leads to smaller starch granules, but the timing of the water stress is important to the distributions (Beckles and Thitisaksakul, 2014). While there was a significant difference in the amount of precipitation between the years, a plant physiological response to drought stress was not seen in 2009. However, a greater amount of smaller granules was observed in 2009 so it may have had a similar impact as a drought stress. The amylose content was slightly lower in the drier growing season which is a similar response to drought stresses observed in wheat (Singh et al. 2008) and rice (Gunaratne et al., 2011). The M_w of the starches from 2008 was larger than 2009 but the chain length distribution of the amylopectin was not altered. Previous studies have demonstrated changes in the chain length distribution due to temperature variation (Shi et al., 1994). The warmer growing season also produced similar findings to gelatinization onset temperature increases as was found by Shi et al. (1994) in wheat. Due to the genetic diversity in sorghum the two hybrids were significantly different for many starch chemistry attributes. These differences were within the normal range of variation for sorghum so few conclusions can be made about the genetic contribution without expanding to a larger sample set.

Generally as the kernels advanced in maturity the volume percent of A-type granules increased, whereas the volume percent of B-type and C-type granules decreased. Early in development (10 DAA and 14 DAA) the size range of 5-10μm had the greatest volume, but by 17 DAA the group with the largest volume was 10-20μm and remained the largest throughout the rest of development. The size groupings 20-30μm and >30μm increased in volume percentage throughout development with little change after 42 DAA. This is more than likely due to starch granules early in development continuing to increase in size to represent the A-type population at advanced maturity stages (Bechtel et al., 1990; Bechtel and Wilson, 2003).

The trend of increasing amylose content as the grain matures has also been found in both wheat (Morrison and Gadan, 1987) and barley (McDonald et al., 1991). The increasing amylose

content also corresponds to a decrease in the crystallinity of the starch as well as the reduction in the lamellar periodicity peak intensity. The increase of amylose content in the amorphous region of the starch appears to disrupt the lamellar periodicity altering the structure of the starch granule.

The gelatinization attributes for the mature (harvestable moisture content) sorghum in this study are similar to previous studies. Beta and Corke (2001) reported gelatinization temperatures and enthalpies on ten sorghum starches. The Tp ranged from 68.0°C to 71.0°C and ΔH ranged from 7.5 J/g to 9.8 J/g. The increased ΔH values along with the greater amount of crystallinity associated to samples early in development suggests that the starch structure is closer to a perfect crystal then degrades over the course maturation either by physical (dehydration) or enzymatic processes. This phenomena has also been demonstrated in potato starches throughout development (Protserov et al., 2000)

Relationships among starch properties and functionality

The starch physical and chemical properties analyzed showed some significant correlations to each other and to the functional properties of the starch (Table 3.7). The starch granule size distribution was highly correlated with the amylose content. The A-type granule groupings were positively correlated with amylose ($r = 0.672$), conversely the B-type and C-type granule groupings were negatively correlated ($r = -0.406$ and $r = -0.788$ respectively). The more extensive breakdown of granule sizing showed that granules in the 10-20μm and 20-30μm ranges were positively correlated to amylose content, but the distribution of >30μm were not significantly correlated. The smaller size groupings were all negatively correlated to amylose content. In wheat starch A-type granules have been shown to have higher amylose content values than the B-type granules when separated from each other (Peng et al., 1999). The starch granule size distributions were also correlated with the amylopectin branch chain distributions. A-type granules were positively correlated with branch chains DP6-12 and DP>37 and negatively correlated to DP13-24 and DP25-36. B-type granules were positively correlated with DP13-24 and C-type granules were positively correlated with DP25-36. Li et al. (2001) found that in barley starch size distributions were correlated to amylopectin branch chain ratios. Amylose content was positively correlated to DP6-12 and DP>37 branch chains and negatively correlated to DP25-36. Previous research has shown that as the amylose content increases, the

average amylopectin branch chain length is increased (Cheetham and Tao, 1997). The starch granule size distribution was also highly correlated with the crystallinity determined by WAXS. The smaller granule groupings (<10μm) were positively correlated with crystallinity, whereas the larger granules were negatively correlated with the crystallinity. The WAXS crystallinity was also negatively correlated with the branch chains DP6-12.

The thermal properties of the starch were found to have some correlations with the starch physical and chemical properties. The onset temperature was negatively correlated with the granule size distribution bin of >30μm ($r = -0.382$) and amylopectin branch chains of DP>37 ($r = -0.430$). The amylopectin branch chain grouping of DP13-24 was positively correlated with the onset temperature ($r = 0.579$). The gelatinization property with the highest number of correlations was ΔH. A-type granules were strongly negatively correlated ($r = -0.829$), B-type granules were strongly positively correlated ($r = 0.898$), and C-type granules were positively correlated with ΔH ($r = 0.432$). The only size grouping that was not significantly correlated to ΔH was the 10-20μm. Previously, the starch granule size distribution has been linked to many functional characteristics, including gelatinization attributes (Eliasson and Karlsson 1983 and Chiotelli and Le Meste 2002).The ΔH was also negatively correlated to both the amylose content and the amylopectin side chain group of DP6-12. Fredriksson et al. (1998) observed a negative correlation of amylose content to gelatinization onset and peak minimum temperatures. They also suggested that amylopectin unit-chain distribution was related to many gelatinization and retrogradation properties. The crystallinity of the starch was positively correlated with the onset temperature of gelatinization.

The retrogradation melting properties were also correlated with the physical and chemical properties of the starches. A-type granules were strongly correlated positively for onset temperature and negatively for the retrogradation melting properties were also correlated with the physical and chemical properties of the starches. A-type granules were strongly correlated positively to onset temperature and negatively for ΔH, ($r = 0.614$ and $r = -0.746$, respectively). B-type and C-type granules were correlated negatively for onset temperature and positively for ΔH. The amylose content was positively correlated to the onset temperature of melting for retrograded starches ($r = 0.430$). Amylopectin branch chain distribution groupings of DP13-24 and DP>37 were correlated with the onset temperature. The ΔH of retrograded starches was also correlated to the amylopectin branch chain distributions. The DP6-12 and DP>37 groups were

negatively correlated, the small chains relationship with retrogradation ΔH has been observed in several starches by Kalichevsky et al. (1990). It was noted that starches with shorter chain lengths retrograded less than those with longer chains. However, in this study a negative relationship with DP>37 was also found. The positive correlation to DP13-24 grouping that was observed suggests that the optimal chain length for retrogradation is in that range since short and very long chains are not conducive to recrystallization.

Conclusions

The hybrid type as well as the growing season contributed to differences in the sorghum chemistry and functionality as was expected. Starch granule size distribution had considerable variability throughout kernel development. The chemical properties of the starch changed as a result of the maturity level, while others remained constant throughout development. The thermal properties of the starch were also greatly influenced by the maturity. The varying thermal properties could impact the utilization of the starch if the grain was harvested at that time. Further investigation into starch functionality is needed to examine where early levels of maturity could be utilized. For instance a sweet sorghum stalk is ready for processing before the grain has reached a typical harvestable moisture content will the properties of the immature grain allow for effective conversion to ethanol.

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Table 3.1 Weather Conditions for the respective growing seasons.

	2008 Seneca	2008 TX631*TX436	2009 Seneca	2009 TX631*TX436
Max Air Temp (°C)	29.3	29.2	27.8	27.0
Min Air Temp (°C)	17.1	16.6	15.5	14.8
Total Precip (mm)	796.1	796.1	471.2	480.4
Sol Rad (MJ/m^2)	2124.4	2355.4	2685.9	2771.4
ET Grass(mm)	524.3	576.6	564.6	578.2
Growing Degree Days	4528.8	4970.7	4220.7	4321.1

Table 3.2 Starch properties from growing season.

	Starch Granule Size Distribution										Amylopectin Branch Chain Length				SEC-MALS	
Year	A Granule Vol. %	B Granule Vol. %	C Granule Vol. %	<2µm Vol. %	2-5µm Vol. %	5-10µm Vol. %	10-20µm Vol. %	20-30µm Vol. %	>30µm Vol. %	Amylose Content %	DP 6-12 %	DP 13-24 %	DP 25-36 %	DP >37 %	Mw x10^7 Da	Rg nm
2008	38.83a*	46.00b	15.17b	7.69b	7.48b	21.21b	45.07a	14.59a	3.97a	22.2a	28.7a	54.4a	11.7a	5.3a	8.6b	143.3a
2009	31.46b	49.62a	18.92a	9.31a	9.60a	24.96a	44.10a	9.16b	2.87b	21.4b	28.8a	53.5a	12.1a	5.5a	9.6a	138.0b
LSD	0.52	1.21	1.40	0.48	0.91	0.52	1.82	0.15	0.23	0.31	0.87	1.18	1.03	0.79	0.001	2.9

*Means with identical letters within each variable are not different (P< 0.05)

Table 3.3 Starch properties from Hybrid

	Starch Granule Size Distribution									Amylose	Amylopectin Branch Chain Length				SEC-MALS	
Hybrid	A Granule Vol. %	B Granule Vol. %	C Granule Vol. %	<2µm Vol. %	2-5µm Vol. %	5-10µm Vol. %	10-20µm Vol. %	20-30µm Vol. %	>30µm Vol. %	Content %	DP 6-12 %	DP 13-24 %	DP 25-36 %	DP >37 %	Mw x10^7 Da	Rg nm
Seneca	40.75a*	44.74b	14.51b	7.53b	6.98b	20.14b	47.35a	14.05a	3.94a	21.7a	28.8a	54.2a	11.7a	5.3a	10.8a	152.6a
TX631*TX436	29.54b	50.74a	19.58a	9.48a	10.10a	26.02a	41.81b	9.69b	2.90b	22.0a	28.7a	53.7a	12.1a	5.5a	7.4b	128.8b
LSD	0.52	1.21	1.40	0.48	0.91	0.52	1.82	0.15	0.23	0.31	0.87	1.18	1.03	0.79	0.001	2.9

*Means with identical letters within each variable are not different (P< 0.05)

Table 3.4 Starch properties from maturity date.

	Starch Granule Size Distribution									Amylose	Amylopectin Branch Chain Length				SEC-MALS	
DAA	A Granule Vol. %	B Granule Vol. %	C Granule Vol. %	<2µm Vol. %	2-5µm Vol. %	5-10µm Vol. %	10-20µm Vol. %	20-30µm Vol. %	>30µm Vol. %	Content %	DP 6-12 %	DP 13-24 %	DP 25-36 %	DP >37 %	Mw x10^7 Da	Rg nm
10	4.19j*	54.17c	41.64a	18.77a	22.87a	44.21a	11.26f	1.29i	1.59e	16.3e	28.2a	54.1a	13.0a	4.7a	8.5a	139.2a
14	10.53i	64.86a	24.60b	12.58b	12.02b	37.98b	35.70e	0.82j	0.90f	19.5d	27.8a	55.1a	12.1a	5.1a	9.6a	141.7a
17	16.35h	65.35a	18.30c	10.25c	8.06c	30.54c	49.37bc	1.78h	0.00g	20.6c	28.7a	54.7a	11.5a	5.1a	9.4a	141.5a
21	23.13g	60.38b	16.50cd	9.30c	7.20cd	25.34d	54.98a	3.18g	0.00g	22.4b	28.5a	54.5a	11.8a	5.3a	9.2a	141.5a
24	30.81f	52.43c	16.78cd	9.17c	7.59cd	22.22e	53.01ab	7.78f	0.24g	22.9ab	29.0a	53.9a	11.6a	5.3a	8.9a	140.3a
28	41.11e	44.87d	14.02de	7.40d	6.62cd	18.33f	51.82ab	13.70e	2.13d	23.3a	29.3a	53.8a	11.6a	5.4a	9.0a	140.4a
31	46.23e	41.10e	12.67ef	6.19e	6.48cd	16.76g	49.64bc	16.16d	4.77c	23.3a	28.9a	53.7a	11.8a	5.5a	9.6a	143.6a
35	48.98c	39.04e	11.99ef	5.96e	6.03cd	15.59gh	49.96bc	17.86c	4.60c	23.3a	28.8a	53.5a	11.9a	5.7a	9.2a	140.1a
42	53.92b	35.51f	10.57f	4.79f	5.78d	14.61hi	45.83cd	21.86b	7.13b	22.4b	29.4a	52.9a	11.8a	5.9a	9.6a	142.0a
49	55.77a	33.98f	10.25f	4.61f	5.64d	14.15i	44.33d	23.07a	8.19a	23.2a	28.2a	53.9a	12.0a	5.9a	8.6a	139.0a
56	55.60a	34.23f	10.17f	4.51f	5.66d	14.16i	44.52d	23.08b	8.07a	23.0ab	29.3a	53.4a	11.9a	5.3a	8.5a	138.4a
LSD	1.22	2.85	3.27	1.13	2.14	1.21	4.26	0.35	0.53	0.73	2.03	2.76	2.41	1.86	0.001	6.89

*Means with identical letters within each variable are not different (P< 0.05)

Table 3.5 WAXD crystallinity of a subset of sorghum hydrated sorghum starches spanning the development of the sorghum kernels.

	DAA	% Crystalline
Seneca	10	15.07
	17	13.43
	28	11.76
	35	11.27
	56	11.57
TX631*TX436		
	10	16.56
	17	13.46
	28	12.84
	35	12.14
	56	11.74

Table 3.6 Thermal properties of sorghum starches.

	Gelatinization				Retrogradation				
Year	T_{Onset} (°C)	T_{Peak} (°C)	T_{End} (°C)	ΔH (J/g)	T_{Onset} (°C)	T_{Peak} (°C)	T_{End} (°C)	ΔH (J/g)	% Retrograded
2008	65.1a	69.9a	75.9a	12.69a	44.2a	55.3a	66.5a	3.22a	26.9a
2009	62.5b	66.2b	71.8b	12.13b	44.4a	54.8b	63.1b	2.90b	23.1b
LSD	0.51	0.30	0.68	0.48	0.73	0.52	0.88	0.27	2.46

	Gelatinization				Retrogradation				
Hybrid	T_{Onset} (°C)	T_{Peak} (°C)	T_{End} (°C)	ΔH (J/g)	T_{Onset} (°C)	T_{Peak} (°C)	T_{End} (°C)	ΔH (J/g)	% Retrograded
Seneca	64.3a	68.8a	74.2a	11.75b	44.0a	54.6b	65.1a	2.76b	23.7b
TX631*TX436	63.2b	67.4b	73.5b	13.07a	44.5a	55.2a	64.5a	3.36a	26.3a
LSD	0.51	0.30	0.68	0.48	0.73	0.52	0.88	0.27	2.46

	Gelatinization				Retrogradation				
DAA	T_{Onset} (°C)	T_{Peak} (°C)	T_{End} (°C)	ΔH (J/g)	T_{Onset} (°C)	T_{Peak} (°C)	T_{End} (°C)	ΔH (J/g)	% Retrograded
10	65.1a	69.0a	76.2a	13.76c	42.0c	54.5bc	65.9ab	3.67ab	26.9ab
14	64.0a	67.6cd	73.0bc	16.06a	42.2c	53.9c	64.3bc	3.93ab	24.6abc
17	64.5a	68.0bcd	73.0bc	14.44bc	42.7c	55.0abc	65.1abc	3.55ab	25.0abc
21	65.0a	68.2bc	73.7bc	14.90b	43.3bc	54.1c	64.8abc	4.13a	28.2a
24	64.4a	67.9bcd	72.7c	15.02ab	44.8bc	54.6bc	66.4a	3.37bc	22.4bc
28	64.3a	68.2bcd	74.4b	12.16d	45.1a	56.0a	63.6c	2.83cd	23.2abc
31	64.0a	68.1bcd	74.2bc	11.20d	44.8bc	54.8abc	63.7c	2.81cde	25.1abc
35	62.8b	67.5d	73.3bc	11.95d	45.2a	55.1abc	64.0bc	2.47de	21.1c
42	62.0b	68.4ab	74.5b	8.69e	46.0a	55.9a	64.9abc	2.17e	25.8abc
49	62.7b	67.9bcd	73.3bc	8.85e	45.5a	54.5bc	64.9abc	2.39de	27.7ab
56	62.7b	68.2bcd	74.0bc	9.45e	45.3a	55.6ab	65abc	2.34de	24.8abc
LSD	1.19	0.69	1.58	1.12	1.71	1.22	2.06	0.64	5.77

*Means with identical letters within each variable and study factor are not different ($P < 0.05$)

Table 3.7 Pearson Correlation coefficients for starch properties analyzed.

	Starch Granule Size Distributions									Amylose Content	Amylopectin Branch Chain Dist.				Gelatinization						Retrogradation			
	A Granule	B Granule	C Granule	<2µm	2-5µm	5-10µm	10-20µm	20-30µm	>30µm		DP6-12	DP13-24	DP25-36	DP>37	Mw	Rg	Onset T	Peak T	End T	ΔH	Onset T	Peak T	End T	ΔH
A Granule	1																							
B Granule	-0.8891	1																						
C Granule	-0.7843	0.4133	1																					
<2µm	-0.8853	0.5802	0.9748	1																				
2-5µm	-0.6795	ns	0.9851	0.9218	1																			
5-10µm	-0.9257	0.7716	0.7958	0.8534	0.7240	1																		
10-20µm	0.4349	ns	-0.7751	-0.6672	-0.8316	-0.6714	1																	
20-30µm	0.9467	-0.9106	-0.6491	-0.7860	-0.5213	-0.7879	ns	1																
>30µm	0.7800	-0.8130	-0.4496	-0.6088	-0.3115	-0.5538	ns	0.8877	1															
Amylose Content	0.6722	-0.4056	-0.7876	-0.7895	-0.7591	-0.7494	0.7228	0.5165	ns	1														
DP6-12	0.3337	ns	ns	ns	ns	-0.3936	ns	ns	ns	0.3388	1													
DP13-24	-0.3153	0.3262	ns	ns	ns	ns	ns	-0.3212	-0.3512	ns	-0.4166	1												
DP25-36	ns	ns	0.4976	0.4331	0.5302	0.4375	-0.6030	ns	ns	-0.5050	-0.5605	-0.3222	1											
DP>37	0.3534	ns	-0.5196	-0.5007	-0.5163	-0.3106	0.3490	ns	ns	0.3137	ns	-0.5556	ns	1										
Mw	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	1									
Rg	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.8967	1								
Onset T	ns	ns	ns	ns	ns	ns	ns	ns	-0.3819	ns	ns	0.5791	ns	-0.4303	ns	0.0990	1							
Peak T	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.3918	ns	-0.3217	ns	0.3972	0.6877	1						
End T	ns	-0.3081	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.3467	ns	-0.3701	ns	ns	0.5835	0.9193	1					
ΔH	-0.8294	0.8984	0.4323	0.5870	0.2982	0.6873	ns	-0.8511	-0.8154	-0.3565	-0.3058	ns	ns	ns	ns	ns	ns	ns	ns	1				
Onset T	0.6145	-0.5270	-0.5081	-0.5651	-0.4466	-0.5792	0.3116	0.5724	0.4361	0.4303	ns	-0.6025	ns	0.5550	ns	ns	ns	ns	ns	-0.4916	1			
Peak T	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.3145	0.4138	-0.3113	0.4420	1		
End T	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.4440	0.3623	0.6960	0.6182	ns	ns	ns	1	
ΔH	-0.7457	0.7356	0.4864	0.5874	0.3918	0.6050	ns	-0.7458	ns	-0.3034	-0.3113	0.4716	ns	-0.3318	-0.3648	ns	0.3567	ns	ns	0.7506	-0.5121	-0.3126	ns	1
% Retro	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.4628	ns	0.3542	0.4117	0.4588	ns	ns	ns	ns	0.47

*ns-not significant (P<0.05)

Figure 3.1 Images of Seneca throughout kernel development.

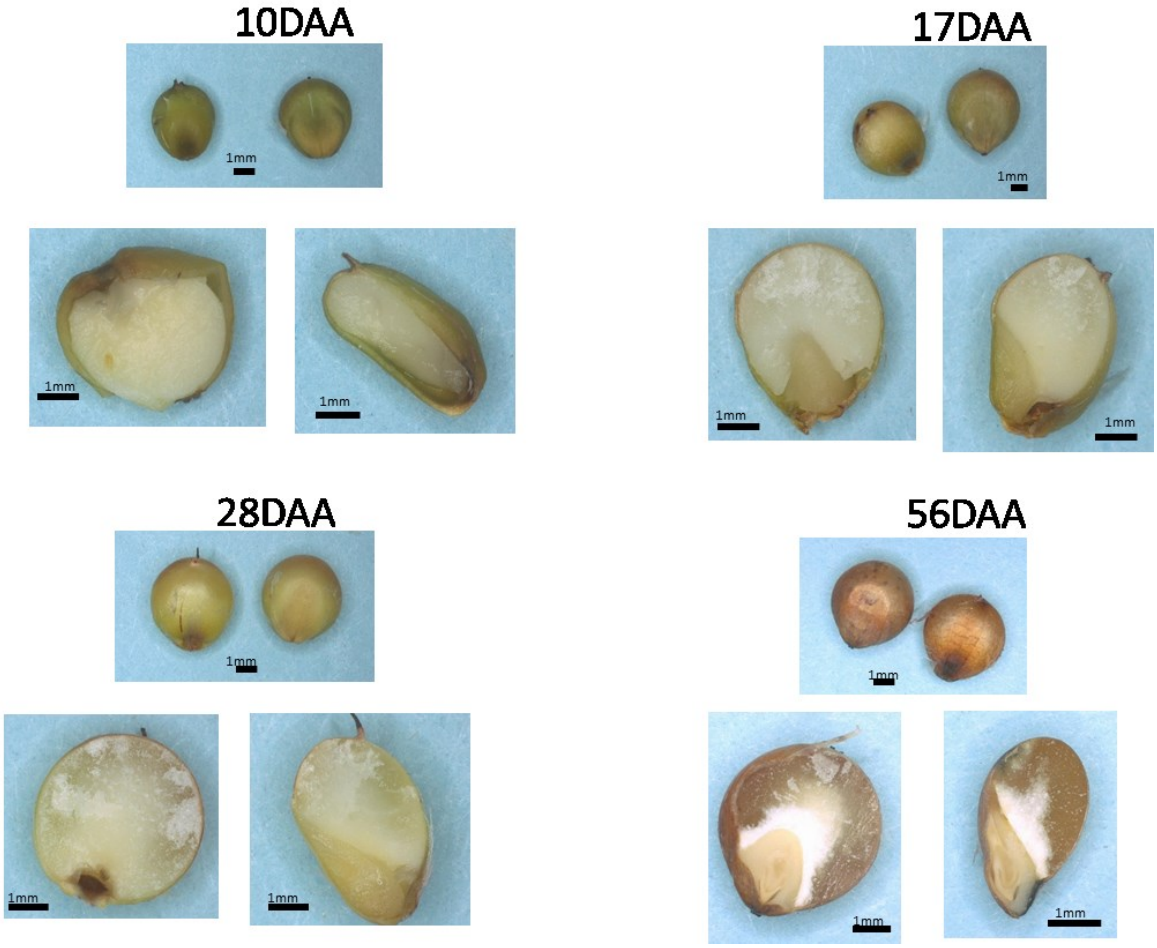


Figure 3.2 Starch granule size distribution throughout kernel development.

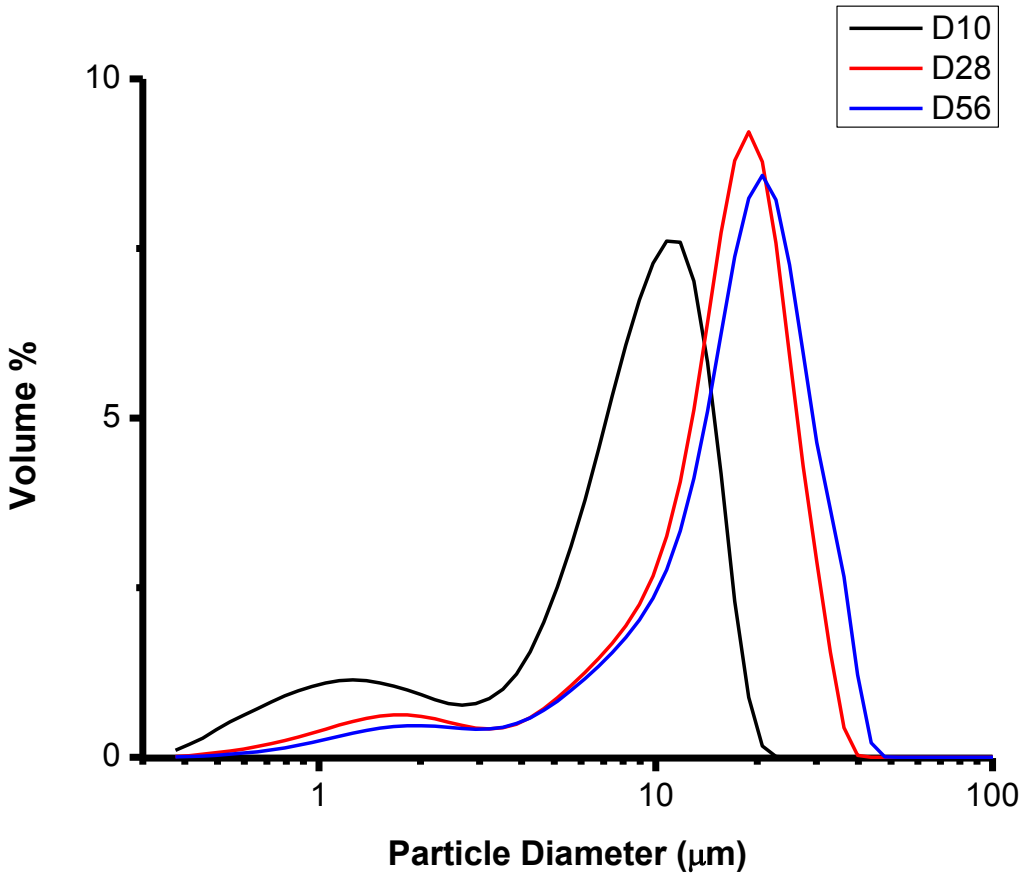


Figure 3.3. Wide-angle X-Ray diffraction patterns for hydrated (A,B) and dry(C,D) starch samples.

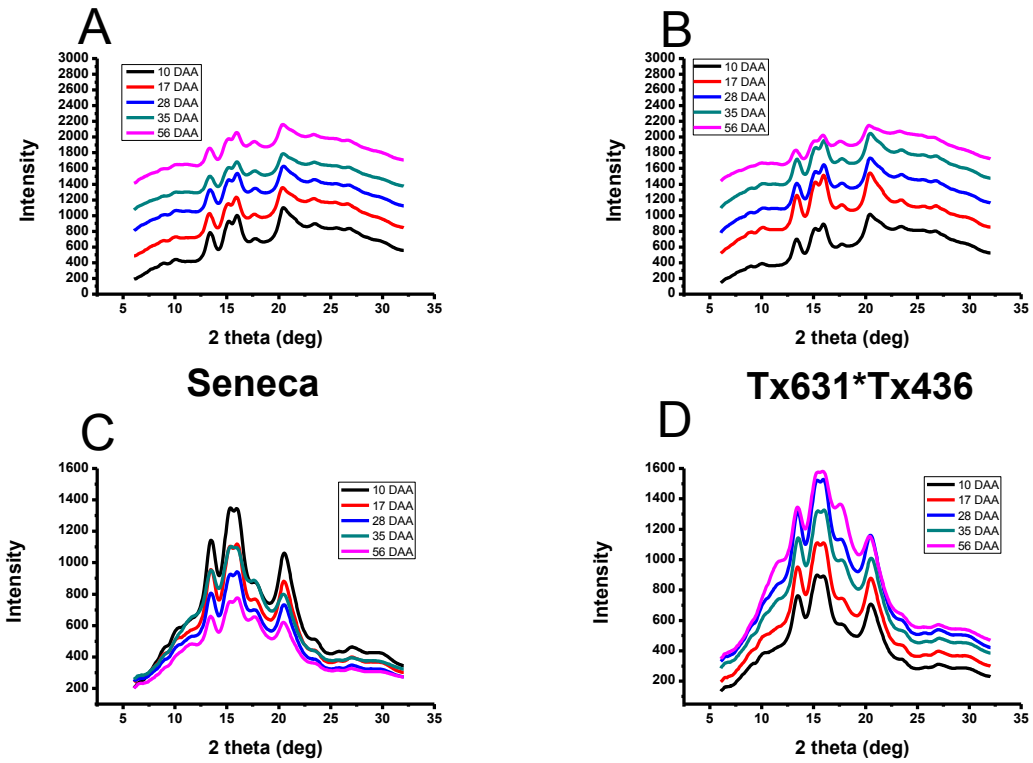


Figure 3.4. Small-angle X-Ray scattering plots spanning the development of sorghum hybrids.

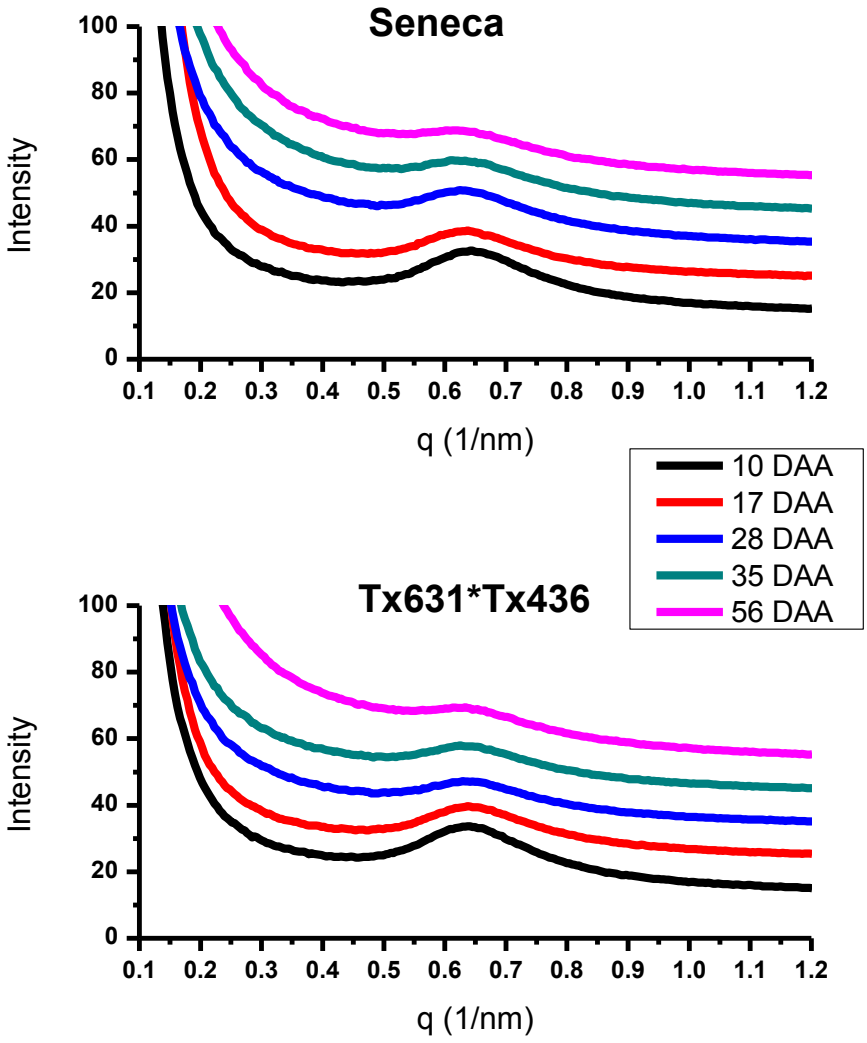
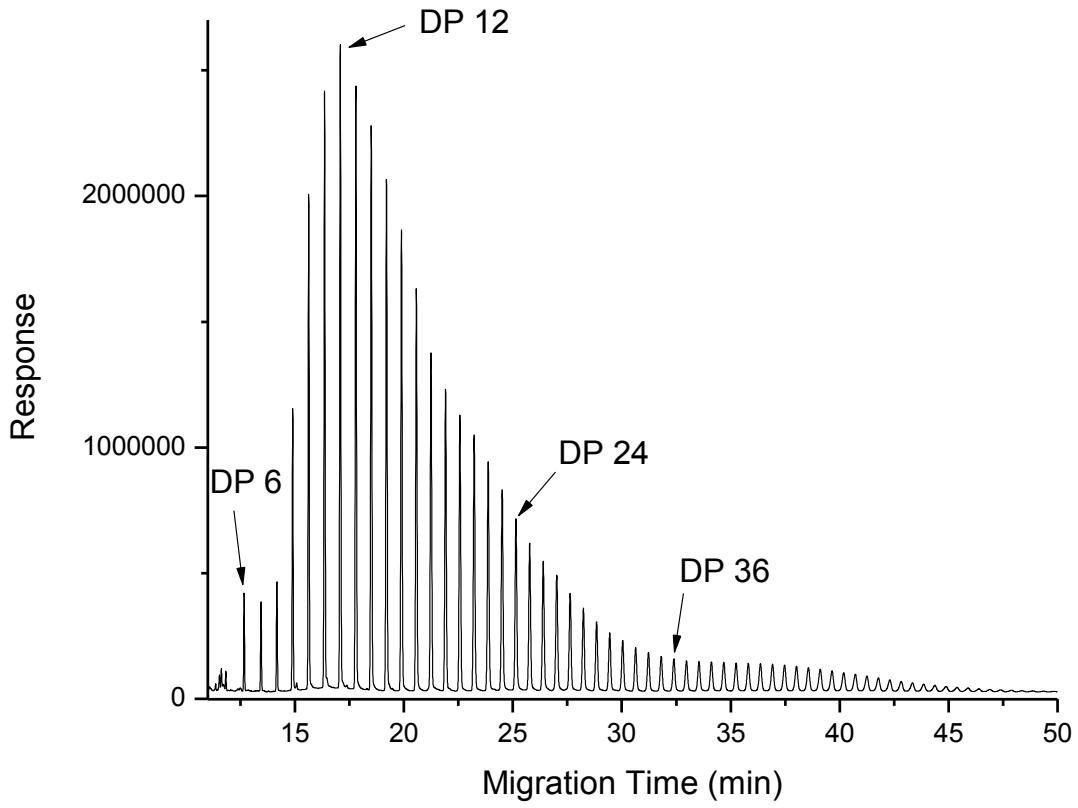


Figure 3.5 An example of the electropherograms used to display amylopectin branch chain length distribution.



Chapter 4 - Sorghum starch and protein digestiblity as affected by growing season, hybrid, and kernel maturity.

Abstract

Sorghum is an important cereal grain in United States agriculture with utilization in the food, feed, and biofuel industries. Compositionally sorghum is very similar to the other cereal grains and can be used almost interchangeably with maize. However the protein and starch digestibility are noted to be lower in sorghum than maize. In this study two sorghum hybrids were grown over two years with sample collection at five separate grain maturity dates spanning the development of the kernel. The protein digestibility and resistant starch contents were measured to determine the effects of kernel maturity. The protein digestibility varied for both growing season and hybrid. The kernel maturity had a notable effect on digestibility with the peak occurring at 17 days after anthesis (DAA) with 82.44% digestible protein. The resistant starch content of isolated starch was found to only vary due to hybrid. There were differences seen in the whole meal resistant starch content, with the greatest resistant starch content (38.93%) found at 35 DAA. The performance of the whole meal in biofuel was predicted with the small scale mashing procedure. The total sugar yield was variable for growing season and hybrid but not maturity suggesting that ethanol yield efficiency would be consistent across kernel maturity. Since the protein and starch digestibility peaks in mid kernel development it may be feasible to harvest grain at earlier maturity to fit animal feeding or biofuel applications.

Introduction

Sorghum (*Sorghum bicolor* [L.] Moench) is the third most important cereal grain grown in the United States behind maize and wheat. Sorghum has a greater heat and drought tolerance compared to maize and is typically grown in the southern Great Plains region of the United States. The agronomic adaptability of sorghum allows sorghum production to be possible with limited or no irrigation and typically is a lower input crop than maize. In the United States sorghum is most commonly used as an animal feed, but has seen increased food utilization due to its gluten-free nature and has also increased in utilization as a feedstock in bioethanol production. However, sorghum has been shown to have a lower starch and protein digestibility than maize (Rooney and Pflugfelder 1986).

The storage proteins in the sorghum endosperm are referred to as kafirins and occur in both polymeric and monomeric forms (Oria et al., 1995). The *in vitro* digestibility of the kafirins found in raw flour or meal varies greatly, though it is typically less digestible than other cereals. Once cooked the protein digestibility typically decreases suggesting that the protein structure is altered by crosslinking (Duodu et al., 2002). The digestibility is also affected by the amount of processing, with the more pure the protein component the higher the digestibility (Duodu et al., 2003). High digestible mutants have been identified with misshapen protein body structures (Weaver et al. 1998; Oria et al. 2000; Tesso et al. 2006) suggesting that the physical properties of the kafirins is also important to accessibility to proteases. Oria et al. (1995) studied the digestibility on developing sorghum grain and found that digestibility was lower at final maturity than throughout development.

The starch in sorghum consists of two types of glucose polymers, amylose and amylopectin, bundled into discreet granules. The starch typically accounts for 55-75% of the kernel by weight (Waniska and Rooney, 2000). Starch is important to the food, feed, and ethanol industry because it can be digested to glucose which serves as an energy source for animal nutrition and the raw material for alcohol fermentation by yeast. The utilization of sorghum starch requires many enzymes (α- and β-amylases, isoamylase, amyloglucosidase) to digest starch to glucose and the enzyme efficiencies are related to many factors including; starch and protein structure (Rooney and Pflugfelder, 1986), particle size of flour (Mahasukhonthachat et al, 2010), and inhibition of enzymes by intrinsic grain components (Mkandawire et al., 2013).

The objectives of this study were to 1) determine the digestibility of sorghum protein and starch as affected by growing season, hybrid, and kernel maturity and 2) determine relationships between starch chemistry attributes and starch functionality.

Materials and Methods

Sorghum samples

A subset of samples was taken from the grain collected and processed in Chapter 3 of this dissertation. The sorghum hybrids "Seneca" and "TX631*TX436" were grown during the summers of 2008 and 2009. Five collection dates that span the kernel development: 10 days after anthesis (DAA), 17DAA, 28DAA, 35DAA and 56DAA were used due to their variation for starch properties and representation of key maturity times. Samples for whole meal analysis were separated from glumes and freeze dried. The freeze dried grain was ground with an Udy mill (Udy Corp., Fort Collins, CO) with a 0.5mm screen. Starch was isolated from decorticated grains also ground with an Udy mill with the sonication procedure of Park et al. (2006).

Protein analysis

Total protein content of the whole grain meal was determined using an N combustion method (AACC method 46-30.01) (AACC International, 2012a) using a LECO FP-528 Nitrogen Determinator (St. Joseph, MI). Nitrogen values were converted to protein by multiplying by 6.25. The modified pepsin method described by Mertz et al. (1984) was used to determine protein digestibility with the digested residues analyzed by N combustion. Samples with non-protein nitrogen removed by trichloracetic acid (TCA) washing (Landry et al. 2000) were also digested.

Starch analysis

Total starch content of the whole meal was analyzed using the Total Starch assay (K-TSTA, Megazyme International, Bray, Ireland) (AACC method 76.13) (AACC International, 2012b). The resistant starch content of both whole meal and isolated starch was found using the Resistant Starch assay (K-RSTAR, Megazyme International, Bray, Ireland) (AACC method 32-40.01). The solubilized (non-resistant) starch content was measured and the resistant starch

pellet was stored for future analysis. The resistant starch content was calculated by subtracting the solubilized starch from the total starch content present in the samples.

The effectiveness of the sorghum meals for ethanol production was measured using the small scale mashing procedure by Zhao et al. (2009). Briefly, 300mg of whole sorghum meal was placed into a 2mL centrifuge tube and one mL of diluted amylase solution (equivalent to 20µl of Liquozyme SC DC (Novozymes North America, Franklinton, NC) per 30 g of sample) was added. The starch slurry was heated at 86 °C for 90 minutes with occasional vortexing. Samples were allowed to cool for five minutes and 50µL of 2M sodium acetate buffer, pH 4.2, was added along with 100µL of diluted amyloglucosidase (equivalent to 200µL of Spirizyme Fuel (Novozymes North America, Franklinton, NC) per 30 g of sample), samples were heated for 90 minutes at 68 °C. Sample tubes were centrifuged at 13,200g for four minutes after cooling at room temperature for 20 minutes. A 200µL aliquot of the supernatant was removed and added to 4.8mL of 0.01M phosphate buffer, pH 10. Samples were filtered with a 0.45µm syringe filter and glucose and maltose were measured using ion exclusion HPLC. A Rezex RCM-monosaccharide column (Phenomonex, Torrance, CA) was used with a mobile phase of 0.6mL/min deionized water. Column temperature was maintained at 75°C and elution was monitored with a refractive index detector (Agilent 1260, Agilent Technologies, Santa Clara, CA) Chromatograms were integrated in Chemstation software.

Statistical Analysis

A completely randomized design was used for all tests with samples in duplicate. Statistical software (SAS v 9.4, SAS Institute, Cary, NC) was used to make Least Significant Difference (LSD) determinations. PROC CORR was used for correlation analysis among the digested properties as well as to the starch properties analyzed in Chapter 3

Results and Discussion

Protein Analysis

The protein content in the sorghum whole meal varied throughout kernel development (Table 4.1). There was significant variation due to growing season, hybrid, and DAA. The samples grown in 2009 had an average protein content of 10.87% with 2008 samples averaging 10.72%. Seneca had a greater protein content across all seasons and DAAs. The samples

exhibited a higher protein content early in development and decreased throughout development even into kernel drydown. The 56 DAA samples had a protein content of 9.86% or approximately 22% less protein than the 10 DAA which had a protein content of 12.6%. Since protein content values are the result of the quantification of nitrogen present then multiplying by a factor (6.25 for sorghum flour) the calculated protein content may be slightly flawed. It has been shown in developing wheat grains that the non-protein nitrogen content is highest early in development and decreases through maturation. The non-protein nitrogen consists primarily of free amino acids (Jennings and Morton, 1963). The prewashing of the sorghum meals with TCA allowed for the removal of the non-protein nitrogen, thus a protein content determination would be more accurate for true protein. Similar trends to the native protein content were observed in the TCA washed meal with the values being considerably lowered (Table 4.1). The 2009 growing season produced meal with a higher protein content, Seneca was higher than Tx631*Tx436, and the protein content was greatest at 10 DAA. Interestingly, the TCA washed protein content for the samples from 17, 28 and 35 DAA were not statistically different and upon kernel drydown the protein content decreases. The high values for protein content in the 10 DAA sample may be explained by lower levels of starch thereby reducing the dilution of the protein increasing its relative protein content and also the nitrogen found the chlorophyll present in the pericarp tissue. The amount of nitrogen removed by the TCA washing showed no differences for growing season or hybrid but was greatest in the 10 DAA sample and declined as the kernel matured. This suggests that early in development there is a considerable amount of non-protein nitrogen present and as the plant is processing the stockpile of nitrogen into protein and other metabolic pathways.

The digestibility of the protein also displayed significant variation attributed to growing season, hybrid, and DAA (Table 4.1). Growing season 2009 had a higher digestibility than 2008 suggesting environmental influences are partially responsible for the digestibility of the protein. The genetic influence on digestibility was exhibited by the difference due to hybrid, which Tx631*Tx436 was more digestible than Seneca. However, it was again the DAA effect on digestibility that was the most interesting. The protein digestibility was highest midway through kernel development (17 DAA), decreased slightly at physiological maturity (35 DAA) and continued to decrease through kernel drydown. Similar decreases in protein digestibility have been seen as a result of drydown (Oria et al. 1995). The crosslinking of the kafirin proteins as a

result of drydown was thought to contribute to this decrease. The very high values for protein digestibility in the native meal can be partially explained due to their increased level of non-protein nitrogen, but the same trend for digestibility was witnessed in the TCA washed samples (Table 4.1). The 17 DAA had the highest digestibility at 70.39% and the digestibility decreased through maturity and drydown. Overall, the TCA washed meals were less digestible than the native meals demonstrating that the non-protein nitrogen that is removed contributes to some of the increased digestibility in the native meals. Further investigation into the kafirin composition is needed to determine the cause of the increased digestibility in the 17 DAA samples and also confirmation of the protein changes in kernel drydown

Starch Analysis

Starch content of the whole meal exhibited variation due to growing season and DAA but not to hybrid (Table 4.2). The growing season of 2008 had a starch content of 74.85% compared to 69.57% in 2009. The sorghum hybrids were not statistically different for total starch content with both around 72%. The samples from early in development (10 DAA) were the lowest in starch content at 59.33% and increased to 77.42% at 28 DAA then decreasing slightly at physiological maturity and drydown. The starch component of the cereal grains undergoes a rapid increase in quantity between 12 and 35 DAA (Shewry, et al., 2009; Wang et al., 2014). Since sorghum is utilized as human food, animal feed, and in ethanol production the digestibility of the starch is an important quality factor. Resistant starch was determined in both isolated starch and whole meal. The isolated starches only varied in resistant starch due to hybrid, suggesting that after isolation the starch's digestibility behavior is dependent on the genetic variability. The resistant starch varied from 4.35% in 56 DAA to 5.24% in 28 DAA but the values were not statistically separated. The digestibility for whole meal samples was determined to more accurately identify digestibility of material commonly used in food or feed products. In whole meal the resistant starch was considerably higher than in isolated starch. There was more resistant starch in samples grown in 2008 than samples grown in 2009, 33.97% to 29.73% respectively. The hybrids did not have any separation for whole meal resistant starch. The 35 DAA samples had the highest amount of resistant starch at 38.93% with the early DAA samples digestibilities higher. After kernel drydown (56 DAA) the whole meal resistant starch content was 32.72%. The increase in resistant starch contents in whole meal as compared to isolated

starch is expected as many factors regarding whole meal could limit starch digestibility, ranging from granule inaccessibility due to the protein bodies (Ezeogu et al., 2005) to particle size of the meal (Al-Rabadi et al. 2009) to inhibition of amylase activity by intrinsic grain components (Hahn et al., 1984; Mkandawire et al., 2013). Since the resistant starch was lowest in the earlier DAA collection dates it appears that the digestibility limiting factors were not as active or fully formed.

Another test of sorghum starch utilization is the small scale mashing procedure used to predict how sorghum hybrids will perform in ethanol production. After mashing the solubilized sugars were analyzed by HPLC and an example chromatogram can be seen in Fig 4.1. Baseline resolution of maltose and glucose can be achieved along with maltotriose, however in the samples analyzed there was not a maltotriose peak separated. There was a peak corresponding to DP4 and greater sugars but the peak areas were not calculated. The combination of the maltose and glucose amounts was referred to as total sugar yield. The total sugar yield was significantly affected by growing season, hybrid, and DAA (Table 4.2). The samples grown in 2009 had a higher sugar yield than 2008 samples, 73.05% to 70.49%. The hybrid Seneca had a greater sugar yield than Tx631*Tx436. In the DAA samples 17 DAA (70.53%) sugar yield was lower than 35 and 56 DAA (~72.5%) but not statistically separated from 10 or 28 DAA. While the 10 DAA sample was very similar to 56 DAA in sugar yield it should be noted when comparing the absolute values of sugars released the 10 DAA would be less due to a lower total starch content in the whole meal. The total sugar yield correlates highly with ethanol production so it is a useful predictor of performance in the ethanol industry (Zhao et al., 2009). Since the total sugar yield did not vary greatly for DAA this could allow for the selection of optimal timing for ethanol production from sorghums. For instance, if a sweet sorghum was being harvested for its stalk syrup at its optimum time, the starch component would still have value as a feedstock for starch based ethanol without concern for drastic reduction in efficiency.

Relationships among digestibility and chemistry

There were not many significant correlations among the digestibility factors (Table 4.3). The protein content only significantly correlated to TCA washed protein, the amount of TCA removed nitrogen, which was expected, and negatively correlated with total starch content, also an expected relationship. The TCA washed protein content was also negatively correlated with

the total starch content, but was positively correlated with the total sugar yield from the small scale mashing. Protein digestibilities were only correlated to each other. There have been studies that linked protein and starch digestibility, but that relationship was not present in this study. The resistant starch content in isolated starch was negatively correlated with the total sugar yield, which is consistent with the notion that as the more sugars are released the less resistant starch is present.

When the digestibility information was correlated to the starch chemistry and thermal properties found in Chapter 3 some interesting relationships were found (Table 4.4). The protein content from both native and TCA washed were linked to the starch granule size distributions, positive correlations with small granules and negative with larger granules. The protein digestibility was also linked with the granule distribution. The granules from 20-30μm and >30μm were strongly negative with native protein digestibility. The B type starch granules were positively linked to both native and TCA washed digestibility. In starch digestibility the resistant starch from isolated starch was only significantly correlated with the Mw of the starch. The resistant starch from whole meal was correlated with the onset and peak temperatures of retrograded starch. Perhaps the most interesting finding in the starch functionality was the relationships to total sugar yield from small scale mashing. The B granules were negatively correlated to sugar yield along with the ΔH from the thermal studies. Sugar yield was positively correlated with the Mw. This suggests that samples with smaller granules with higher levels of organization were not as easily converted to fermentable sugars.

Conclusions

The sorghum meals and starches studied exhibited large variations in their protein and starch digestibility measurements. Protein digestibility peaked before physiological maturity and decreased through kernel dry down. This may be useful to animal feeding of sorghum grains that get harvested for ensilage or high moisture feeding. The decrease in protein digestibility during kernel drydown needs further investigation to determine the cause of the decrease. The starch in whole meal had less resistant starch early in development, meaning the starches were more digestible, but there is less total starch at this phase so net energy may be very similar. The small scale mashing yielded total sugar yields with only slight variations, this would allow starches from grain to be used from any maturity. Again the overall starch content is lower at

early maturity stages, so the total ethanol production would be lower on an absolute scale but the starches would perform equally on a relative basis.

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Table 4.1 Mean values for protein content and digestibility from growing season, hybrid, and maturity (DAA).

	Protein Content Native %	Protein Digestibility Native %	Protein Content TCA Washed %	Protein Digestibility TCA Washed %	TCA removed Nitrogen %
Year					
2008	10.72b*	72.65b	8.54b	59.93b	0.349a
2009	10.87a	78.60a	8.77a	67.47a	0.336a
LSD	0.10	1.45	0.12	1.21	0.02
Hybrid					
Seneca	11.07a	74.56b	8.88a	63.51a	0.350a
TX631*TX436	10.52b	76.69a	8.43b	63.88a	0.336a
LSD	0.10	1.45	0.12	1.21	0.02
DAA					
10	12.60a	76.66c	9.25a	59.99c	0.537a
17	10.75b	82.44a	8.64b	70.39a	0.337b
28	10.28d	79.56b	8.61b	69.97a	0.264c
35	10.50c	73.09d	8.63b	65.11b	0.299bc
56	9.86e	66.38e	8.14c	55.04d	0.276c
LSD	0.17	2.30	0.12	1.91	0.04

*Means with identical letters within each variable and study factor are not different ($P < 0.05$)

Table 4.2 Mean values for total starch, resistant starch, and total sugar yield from growing season, hybrid and maturity.

	Total Starch	Resistant Starch		Small Scale Mashing
	Whole Meal	Isolated	Whole Meal	Total Sugar Yield[1]
	%	%	%	%
Year				
2008	74.85a[*]	4.87a	33.97a	70.49b
2009	69.57b	4.89a	29.73b	73.05a
LSD	0.68	1.79	2.87	1.16
Hybrid				
Seneca	72.25a	2.92b	31.36a	73.63a
TX631*TX436	72.16a	6.84a	32.34a	69.90b
LSD	0.68	1.79	2.87	1.16
DAA				
10	59.33d	5.15a	27.75c	71.28ab
17	73.59c	4.81a	26.42c	70.53b
28	77.42a	5.24a	33.43b	72.05ab
35	74.87b	4.84a	38.93a	72.51a
56	75.82b	4.35a	32.72b	72.48a
LSD	1.07	2.83	4.54	1.83

[1]Total Sugar Yield is [Glucose+Maltose]/[Total Starch]

[*]Means with identical letters within each variable and study factor are not different (P< 0.05)

Table 4.3 Pearson correlation coefficients for sorghum protein and starch digestiblities.

	Native Protein Content	Native Protein Digestibility	TCA washed Protein Content	TCA washed Protein Digestibility	TCA removed Nitrogen	Total Starch	Resistant Starch, Isolated	Resistant Starch, Whole Meal	Small Scale Mash, Total Sugar Yield
Native Protein Content	1								
Native Protein Digestibility	ns*	1							
TCA washed Protein Content	0.85635	ns	1						
TCA washed Protein Digestibility	ns	0.76211	ns	1					
TCA removed Nitrogen	0.88481	ns	0.51709	ns	1				
Total Starch	-0.86771	ns	-0.73821	ns	-0.77268	1			
Resistant Starch, Isolated	ns	ns	ns	ns	ns	ns	1		
Resistant Starch, Whole Meal	ns	ns	ns	ns	ns	0.45086	ns	1	
Small Scale Mash, Total Sugar Yield	ns	ns	0.55017	ns	ns	-0.44883	-0.47461	ns	1

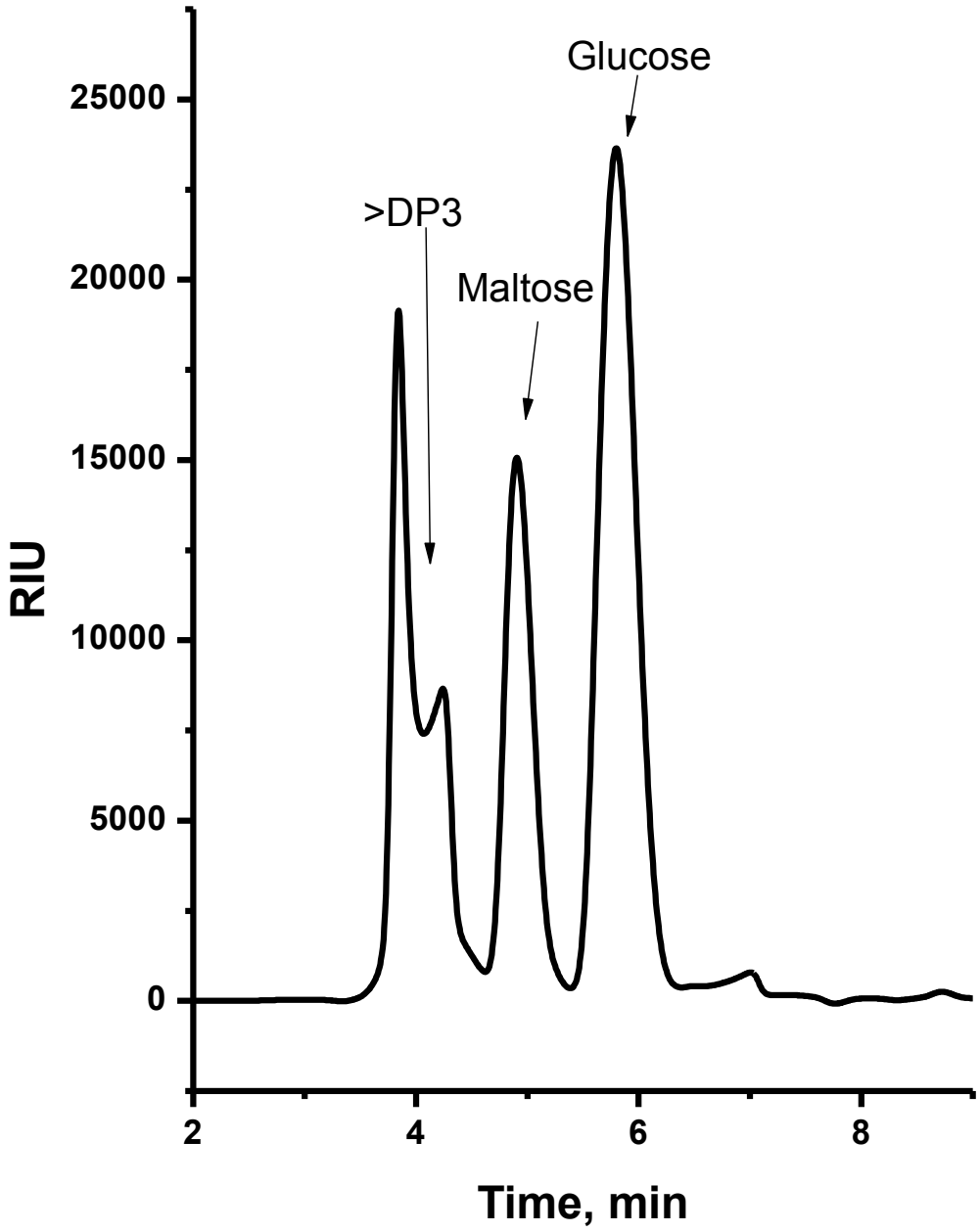
*ns-not significant ($P<0.05$)

Table 4.4 Pearson correlation coefficients for sorghum protein and starch digestibilities to starch chemical and thermal attributes.

	Native Protein Content	Native Protein Digestibility	TCA washed Protein Content	TCA washed Protein Digestibility	TCA removed Nitrogen	Total Starch	Resistant Starch, Isolated	Resistant Starch, Whole Meal	Small Scale Mash, Total Sugar Yield
A Granule	-0.5897	-0.6046	ns	ns	-0.7050	0.5401	ns	ns	ns
B Granule	ns*	0.6840	ns	0.5308	ns	ns	ns	ns	-0.4536
C Granule	0.7835	ns	0.5655	ns	0.7887	-0.8474	ns	ns	ns
<2µm	0.7681	ns	0.5392	ns	0.7867	-0.7863	ns	ns	ns
2-5µm	0.7767	ns	0.5715	ns	0.7722	-0.8719	ns	ns	ns
5-10µm	0.6437	ns	ns	ns	0.8256	-0.5869	ns	ns	ns
10-20µm	-0.7599	ns	-0.4493	0.4951	-0.8546	0.8134	ns	ns	ns
20-30µm	-0.4722	-0.7715	ns	ns	-0.5376	ns	ns	ns	ns
>30µm	ns	-0.8449	ns	-0.5643	ns	ns	ns	ns	ns
Amylose Content	-0.7535	ns	-0.4783	ns	-0.8174	0.7172	ns	ns	ns
DP6-12	ns	ns	ns	ns	-0.5929	ns	ns	ns	ns
DP13-24	ns	ns	ns	ns	ns	ns	ns	ns	ns
DP25-36	0.5499	ns	ns	ns	0.7143	-0.5185	ns	ns	ns
DP>37	-0.5798	ns	-0.5665	ns	-0.4505	0.5981	ns	ns	ns
Mw	ns	ns	ns	ns	ns	ns	-0.5025	ns	0.4837
Rg	ns	ns	ns	ns	ns	ns	ns	ns	ns
Gel. Onset T	ns	ns	ns	ns	ns	ns	ns	ns	ns
Gel.Peak T	ns	ns	ns	-0.4953	ns	ns	ns	ns	ns
Gel. End T	ns	ns	ns	-0.5875	ns	ns	ns	ns	ns
Gel. ΔH	ns	0.6487	ns	ns	ns	ns	ns	ns	-0.5470
Ret. Onset T	-0.5636	ns	ns	ns	-0.5893	0.5728	ns	0.4898	ns
Ret. Peak T	ns	ns	ns	ns	ns	0.4561	ns	0.4912	ns
Ret. End T	ns	ns	ns	ns	ns	ns	ns	ns	ns
Ret. ΔH	ns	ns	ns	ns	0.5293	ns	ns	ns	-0.5131
% Retro	ns	ns	ns	ns	ns	ns	ns	ns	ns

*ns-not significant ($P<0.05$)

Figure 4.1 Example chromatogram from samples after small scale mashing.



Chapter 5 - The influence of genetic and environmental factors on sorghum grain chemistry and digestibility.

Abstract

Sorghum is an important cereal crop for both animal feed and biofuel production in the United States. The genetic diversity coupled with the wide range in growing conditions allows for the potential of great variation in sorghum grain quality. A diverse set of 19 sorghums was grown in three locations in Kansas to evaluate the genetic, location, and genetic x location effect on grain quality attributes. The physical characteristics of the grain (size and hardness) were greatly affected by genotype, location, and their interaction. The chemical components and functional properties were also affected by genotype and location. The amylose content varied from 19.2% to 30.7% and was also variable due to growing location. The starch granule size distribution exhibited little variation due to location, but was affected by the genotype. Protein content ranged from 11.09% to 15.17% and digestibility ranged from 45.58% to 62.05% due to genotype. The small amount of interaction between genotype and location for the protein content and digestibility allows for selection of the desired genotype and location without a wide variation due to their combination.

Introduction

Sorghum is a widely grown cereal grain native to the arid regions of Sub-Saharan Africa. In many parts of the world sorghum is a dietary staple for humans, however in the United States it has commonly been used as animal feed. Recently sorghum has become a more popular human food in the United States due to the increase in gluten-free food consumption. Sorghum has also experienced an increase in utilization due to the fuel ethanol industry.

Sorghum is very genetically diverse resulting in large variation in both agronomic attributes and grain quality attributes. This genetic diversity results into sorghums being traditionally being classified into five basic races (Bicolor, Guinea, Caudatum, Kafir, and Durra) as well as ten intermediate races which are combinations of the basic races (Harlan and de Wet, 1972). The races were classified based on their panicle shape and size as well as their physical grain characteristics. The races were also geographically separated in their domestication/origination leading to their unique appearances. Recent studies have begun classification of sorghums based on their genetics. Casa et al. (2008) identified ten subpopulations using an association mapping panel, Brown et al. (2011) studied genetic relationships in comparison to phenotypic racial groupings, and Sukamaran et al. (2012) evaluated grain quality in five genetic subpopulations that corresponded to race groups.

Advancements in the genetic screening and improvement of sorghum have led to the discovery of mutants with altered properties including increased protein and starch digestibility. The increased protein digestibility mutant is the result of misshapen protein bodies (Weaver et al., 1998; Oria et al., 2000; Tesso et al., 2006). A single point mutation in the kafirins has been linked to the misshapen protein bodies (Wu et al., 2013). A mutant allele for the gene encoding for pullulanase, an enzyme the starch metabolic pathway, allows for increased starch digestibility regardless of genotypic background (Gilding et al., 2013). The genetic improvements in sorghum are only part of the resulting grain quality with environmental factors also influencing the grain quality.

Due to its tolerance to heat and drought stress sorghum has been traditionally grown in the semi-arid parts of the southern Great Plains region of the United States. In recent years increased sorghum production has also been found in North Carolina and Arkansas, regions that receive more rainfall than the plains. Heat and drought stresses have been shown to drastically affect the grain quality in other cereals such as wheat, barley, and maize. Protein formation and

crosslinking in wheat and maize has been found to be altered by both heat and drought stresses (Daniel and Triboi, 2002; Ober et al., 1991; Ashraf, 2014). Starch synthesis is also greatly affected by both heat and drought stress (Thitisaksakul et al., 2012; Ashraf, 2014). However, the environmental effects on a diverse set of sorghum genotypes have not been extensively studied.

The wide range of sorghum growing regions combined with the genetic diversity of sorghum poses significant challenges in maintaining consistent quality sorghums across growing regions. Thus, the objectives of this research is to evaluate the effect of diverse genotypes and growing locations on the chemical and physical properties of sorghum grain, identify genotypes that are consistent across growing locations, and link environmental factors to sorghum quality attributes.

Materials and Methods

Grain sorghum samples

A genetically diverse set of 19 sorghums were grown in three locations spanning the growing regions of Kansas. The sorghums were grown in the 2013 in Manhattan, KS (eastern region), Hays, KS (central region), and Colby, KS (western region). The weather conditions throughout the growing seasons were monitored and data collected via the Kansas Mesonet. The sorghum samples were selected from the larger association mapping panel created by Casa et al. (2008) based on their protein digestibility values ranging from high to low digestibility. Table 5.1 shows the sample set along with their traditional race and genetic groupings.

Sample processing

Grain was harvested and mechanically cleaned and analytical procedures performed on the whole grain. The samples were then divided for analysis of protein and starch properties. The samples for protein analysis were ground into whole meal using an UDY mill (Udy Corporation, Fort Collins, CO) equipped with a 0.5 mm screen. For starch isolation, grain samples were first decorticated (~15% removal) using a TADD (Venables Machine Works) and then decorticated grain meal was produced similarly to the whole grain meal. Starch was then isolated from the decorticated meal by using the sonication method of Park et al. (2006).

Whole kernel analysis

The sorghums were analyzed by NIR spectroscopy at Texas A&M University for the starch, fat, fiber, and ash components. A detailed description of the NIR instrumentation can be found in Dykes et al. (2014).

The kernel size (diameter and weight) and hardness values were found using a single kernel characterization system (SKCS 4100, Perten Instruments) controlled by SKCS for Windows software (Version 2.1.0.1) using 100 kernels per sample (Bean et al., 2006).

Chemical analysis of sorghum meal and starch

Total protein content of the whole grain was determined using an N combustion method (AACC method 46-30.01) (AACC International, 2012) using a LECO FP-628 Nitrogen Determinator (St. Joseph, MI). Nitrogen values were converted to protein by multiplying by 6.25. The modified pepsin method described by Mertz et al. (1984) was used to determine protein digestibility with the digested residues analyzed by N combustion.

Starch granule size distribution was found using laser diffraction sizing. Samples were allowed to hydrate in an aqueous solution (1% sodium azide) overnight at 4° C before being analyzed using a single wavelength LS 13 320 Particle Size Analyzer (Beckman Coulter, Miami, FL) equipped with the aqueous liquid module and autosampler. Data were collected as volume percent measurements and binned with the common size groupings: A-type (>15µm), B-type (5-15µm), and C-type (<5µm). An additional set of size groupings (<2µm, 2-5µm, 5-10µm, 10-20µm, 20-30µm, and >30µm) was also utilized for a more extensive analysis of size information.

Amylose content of the starch was found using the 96-well microplate dual-wavelength iodine method developed by Kaufman et al. (2015) (Chapter 2).

Statistical analysis

Samples were grown in duplicate in the field plots and all subsequent analyses utilized field replicates. Laboratory duplicates were used for all chemical analyses. Data were analyzed using SAS version 9.4 (SAS Institute, Cary, NC). Proc GLM was used for ANOVA analysis and means were separated using Fisher's Least Significant Difference. Proc Corr was used to determine Pearson's correlation coefficients between the sorghum properties.

Results and Discussion

Growing Conditions

The three growing locations across Kansas provided three unique weather conditions across the entire growing season (Table 5.2). The easternmost location (Manhattan) was coolest for both air and soil temperatures. Hays had the warmest average air temperatures and Colby had the highest soil temperatures. Manhattan also received the greatest amount of total precipitation at 327.9 mm of rainfall, nearly 66% greater than Colby and 21% greater than Hays. Colby had the lowest average relative humidity followed by Hays then Manhattan. The average maximum wind speed was found in Hays at 11.66 m/sec. The highest temperatures and wind speed contributed to Hays having the largest calculated evapotranspiration of the three locations. The three locations unique weather conditions led to differences experienced in the grain physical and chemical properties; however soil properties were not tested so variability cannot be fully assigned to the weather conditions. The location effect discussed in the ANOVA will encompass all variables associated with the growing location.

Genetic and Location Effects

The ANOVA of the data showed that all of the whole kernel NIR measurements and Single Kernel Characterization measurements were significantly affected by the genetic, location, and the genetic x location interaction (Table 5.3). However, the interaction contributed a small relative amount to the overall variance. The chemical components were not as affected by the location or interaction. Amylose content and starch granules >30µm were the only starch components affected by location. The granule grouping 10-20µm was significant for genetic x location interaction. The entire set of starch components was significant for the genetic factor. The protein and protein digestibility were significantly affected by both the genetic and location factors. However, there was no significant genetic x location interaction for either protein or protein digestibility. Since the interaction of genetic x location was not a major contributor to the overall variance, only the effects of genetic and location on the physical and chemical components will be discussed. ANOVA demonstrates that the sorghum grain quality factors are significantly affected by the genetic and location components, thus consideration for the components in needed when evaluating sorghums for potential end-use.

Whole Kernel Analysis

The fat, fiber, ash, and starch contents were measured by NIR and exhibited a wide variation due to the sorghum genotype across all locations (Table 5.4). The fat content varied from 2.2% in SC1056 to 4.6% in SC471. The fiber, ash, and starch did not exhibit as great of range in variation as the fat. Fiber varied from 1.61% to 2.03%, ash varied from 0.81% to 1.06% and starch varied from 67.1% to 70.7%. The range for the attributes is within the reported ranges for proximate analysis (Waniska and Rooney, 2000). The large ranges for the chemical composition of sorghum are expected due to the genetic diversity of the sample set. The physical components of the sorghum kernels also exhibited a large variation due to genotype. Kernel hardness varied from 67.5 hardness index (HI) to 101.4 HI. The hardness of the kernels is an important trait due to its importance in processing of the sorghum. The sorghums also exhibited a large variation in both kernel weight and diameter. SC391 was the largest seeded genotype with a 2.81mm kernel diameter and weighing 36.8mg. The genotype SC1104 was less than half of the weight of SC391 at 18.0mg.

The location effects on the NIR proximate analysis and physical attributes showed mean separation across genotypes (Table 5.5). The sorghums grown in Hays, KS had the largest amount of fat and fiber while exhibiting the largest and hardest kernels. Manhattan, KS grown sorghums were highest in starch and ash content while having the softest kernels. The size (weight and diameter) of kernels in Manhattan were equal to that of the Hays location. The starch content of Hays and Colby were equal (68.5%), however every other attribute showed Colby grown sorghums statistically separated from the high and low values. The difference in the attributes of sorghum for locations demonstrates that growing location could be important if an attribute is needed for a specific end product.

Starch Analysis

The amylose content of the starch was heavily influenced by the genotype with most sorghums grown being “normal” type starches which contain both amylose and amylopectin (Table 5.6). The amylose content ranged from 19.2% to 31.4% in BTx Arg-1 and SC471 respectively. The BTx Arg-1 genotype could possibly be considered a heterowaxy sorghum due to its lower amylose value. The growing location also had a significant effect on the amylose content. All three locations were statistically different in their mean amylose value across

genotypes (Table 5.7). Manhattan exhibited the highest amylose content and Hays was the lowest. This difference due to locations suggests that environmental factors can affect the amylose:amylopectin ratio. The environmental effect on amylose content has been found in other cereal grains such as wheat (Singh et al., 2008) and rice (Gunaratne et al., 2011).The ratio of amylose:amylopectin is important to many functional properties of the starch, such as gelatinization profile (Fredriksson et al., 1998).

Another important attribute of the starch in food, feed, and industrial systems is the starch granule size distribution. There was a large variation in size distributions due to genotype (Table 5.6). The distributions for A-type granules ranged from 35.4 Volume % to 57.1 Volume % in BTx Arg-1 and SC489 respectively. Conversely, BTx Arg-1 had the highest values for both B-type and C-type granules while SC489 had the lowest for B-type and C-type. BTx Arg-1 exhibited the highest value for very small granules (<2μm) and SC108 had the greatest level of very large granules (>30 μm). The starch granule size distribution did not experience many differences due to growing location (Table 5.7). One small difference was found in the >30μm grouping with Colby being significantly separated from Manhattan. This may suggest that environmental influences affect the distributions slightly but the overall differences in conditions at our locations were not great enough to illicit more distribution variation. Starch granule size distributions have been linked to other starch properties such as amylose content (Chapter 3) as well as gelatinization behaviors (Eliasson and Karlsson, 1983; Chiotelli and Le Meste, 2002).

Protein Analysis

The sorghum whole meal protein content was influenced by both the genotype and the growing location. A wide range of protein content was found due to the genotype, ranging from 11.09% in BTx399 to 15.17% in SC1277 (Table 5.6). Since there is a wide range in genetic diversity with this sample set the range in protein content values due to genotype was expected and fell within the normal range of protein content for sorghums (Waniska and Rooney, 2000). The growing locations also had significant mean separation with Manhattan and Colby (13.04% and 12.98%, respectively) greater than Hays (12.55%) (Table 5.7). The location or environmental effect on protein content has also been reported in many cereal grains including wheat (Hasniza et al., 2014), oat (Doehlert and McMullen, 2000), rice (Champagne et al., 2004, and sorghum (Wu et al., 2008). It should be noted that only the weather conditions were

collected for the growing locations, the soil conditions could also affect the protein content of sorghum (Kaufman et al., 2013). There were only minor differences in the relative protein content of the sorghum genotypes across the locations suggesting that the interaction genotype and location will not result in great differences, i.e. a high protein sorghum grown in one location will be relatively high in another location though the absolute values may be different.

The protein digestibility also displayed a wide range of values due to both the genotype and the location (Tables 5.6 and 5.7). The digestibility ranged from 45.58% in SC645 to 62.05% in SC471. The protein digestibility difference due to genotype was expected and further investigation into the protein chemistry, such as kafirin composition and size distribution, is needed to identify the cause of this variation. The kafirin composition is thought to greatly influence the protein digestibility (Oria et al., 1995). Manhattan grown sorghums exhibited the lowest mean digestibility at 52.22% with the Hays location the highest at 57.38%, Colby's mean digestibility was statistically separate from both at 55.32%. The digestibility followed a similar trend as protein content in that a genotype that ranks high in digestibility at one location was also found to be high at another location. The protein digestibility of sorghum was not found to be influenced by soil properties as the protein content was (Kaufman et al., 2013), therefore other environmental conditions are responsible for the variation in digestibility due to location. However, relationships with specific environmental conditions were difficult to conclude due to the limited number of growing locations.

Relationships among attributes

Significant correlations were observed between some of the sorghum grain attributes (Table 5.8). The amylose content was linked to the size distribution of the starch granules, a negative correlation with small granules as was seen in Chapter 3. The amylose was also negatively correlated to the fat and fiber content and positively correlated with the ash content. Perhaps the most interesting correlations were those of the starch properties to grain hardness. The amylose content and very small starch granules (<2μm) were negatively correlated to hardness while the granule range of 20-30μm was positively correlated. This suggests that the structure of the starch granules may influence the compressive strength of the grain. Interestingly, the protein content was not correlated to grain hardness as has been previously reported (Kaufman et al., 2013). The protein digestibility was positively correlated with the fat

content and negatively correlated with very large starch granules (>30μm), the ash content, and the protein content.

The small number of locations made correlations to environmental conditions unreliable. An expansion in number of growing locations is needed to further identify the environmental relationships to sorghum grain components.

Conclusions

The diverse set of sorghum genotypes and variation in growth conditions led to differences in both the physical and chemical components in the grain. Most of the variation of the sorghum chemical attributes was due to either the genotype or location effect with little interaction observed. The physical grain characteristics such as kernel size and hardness exhibited more variation due to genotype x location interaction. Thus, the variation due to genotype and location will allow for the ability to select the most desirable trait at a specific location.

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Table 5.1 Traditional and genetic classification of sorghums analyzed.

Genotype	Sukamaran group	Traditional race	Casa group	Country of Origin
B OK11	2	kafir	kafir	USA
BTx Arg-1	.	.	.	USA
BTx2752	2	breeding line	kafir	USA
BTx399	2	kafir	kafir	USA
BTx643	3	breeding line	kafir	USA
P898012	5	cultivar	caudatum	USA
RTx430	4	breeding line	milo-feterita	USA
SC1056	.	.	.	Sudan
SC108	3	caudatum	zerazera-caudatum	Ethiopia
SC1104	5	kafir-bicolor	caudatum	Uganda
SC1211	5	kafir-caudatum	guinea-bicolor	C. America
SC1277	.	.	.	.
SC38	1	durra	durra	Ethiopia
SC391	4	caudatum	guinea-caudatum	Egypt
SC414	.	.	caudatum-kafir	Sudan
SC471	.	.	.	India
SC489	1	durra	durra	India
SC628	2	kafir	kafir	S. Africa
SC645	5	kafir-caudatum	caudatum	Uganda

Table 5.2 Key weather observations for the growing locations.

	Air Temperature max °C	Air Temperature min °C	GDD Total	Precipitation total mm	Solar Radiation total MJ/m^2	Soil Temperature 10cm max °C	Soil Temperature 10cm min °C	Relative Humidity avg %	WindSpeed2m max m/s	ET Total mm
Colby	31.24	15.97	1809.65	197.11	2404.80	27.99	22.51	58.26	10.92	745.06
Hays	31.98	17.16	2020.20	270.49	2287.00	26.38	22.18	60.10	11.66	791.73
Manhattan	29.46	16.96	2239.85	327.90	2665.60	24.49	21.95	66.76	8.34	670.00

Table 5.3 ANOVA mean square values testing the effect of the genotype and location on sorghum grain attributes.

		Whole Kernel NIR				Single Kernel Analysis				Starch Granule Size Distribution								Protein Analysis	
Source	**DF**	**Fat**	**Fiber**	**Ash**	**Starch**	**Hardness**	**Wt**	**Dia**	**Amylose**	**A Granule**	**B Granule**	**C Granule**	**<2µm**	**2-5µm**	**5-10µm**	**10-20µm**	**20-30µm**	**>30µm**	**Content**	**Digestibility**
Genotype	18	3.094*	0.084*	0.040*	5.189*	553.111*	0.507*	127.718*	44.524*	144.349*	112.300*	3.096*	1.186*	1.100*	26.474*	29.587*	70.531*	3.359*	7.398*	117.75*
Location	2	11.583*	0.39*	0.126*	18.977*	627.441*	0.040*	13.039*	240.626*	17.937	16.69	0.034	0.116	0.252	0.017	6.968	5.759	6.370*	2.761*	255.84*
Genotype x Location	36	0.093*	0.014*	0.005*	0.686*	29.695*	0.026*	6.252*	6.796	11.559	8.927	0.295	0.204	0.084	2.399	5.659*	5.89	1.342	0.807	17.58
Error	57	0.031	0.001	0.001	0.188	10.275	0.006	1.509	8.27	11.55	7.607	0.586	0.179	0.223	3.29	2.485	4.23	1.337	0.515	14.32

*Mean Square value is significant at ($P < 0.05$)

Table 5.4 Effect of genotype on the mean values of sorghum whole kernel attributes.

	Whole Kernel NIR				Single Kernel Analysis		
Genotype	**Fat**	**Fiber**	**Ash**	**Starch**	**Hardness**	**Weight (mg)**	**Diameter (mm)**
B OK11	2.7f*	1.66j	1.07a	69.9b	78.5fghi	25.4def	2.19cd
BTx Arg-1	4.0c	1.77gh	0.87h	69.5bcd	95.2bc	21.6ij	1.94ef
BTx2752	3.6d	1.93bc	0.93ef	69.0def	101.4a	23.5gh	2.12d
BTx399	3.1e	1.90bc	0.99c	69.3cde	77.8hi	22.8hi	2.18cd
BTx643	4.0c	2.03a	0.91fg	69.7bc	98.4ab	24.8efg	2.19cd
P898012	3.0e	1.80fg	1.05ab	67.9i	67.5j	30.9c	2.68b
RTx430	3.8d	1.94b	0.97cd	68.5gh	87.2e	32.6b	2.61b
SC1056	2.2h	1.73hi	1.04b	69.8bc	77.4i	21.8ij	1.99e
SC108	4.2bc	1.85de	0.88gh	67.6ij	81.3fgh	24.3fg	1.98e
SC1104	2.5g	1.80fg	1.04ab	69.0efg	98.4ab	18.0l	1.88fg
SC1211	2.5g	1.82ef	1.06ab	68.5h	77.9ghi	26.8d	2.63b
SC1277	4.1bc	2.03a	0.91fg	67.1j	95.8bc	26.3d	2.19cd
SC38	4.3b	1.85e	0.86h	69.9b	93.3cd	23.7gh	2.24c
SC391	3.7d	1.72i	0.99c	68.6fgh	75.2i	36.8a	2.81a
SC414	3.0e	1.75hi	1.06ab	68.8fgh	81.5fg	25.8de	2.21cd
SC471	4.6a	1.73hi	0.81i	67.7i	82.0f	20.8jk	2.00e
SC489	4.1bc	1.61k	0.88gh	69.5bcd	96.3bc	21.3j	1.95ef
SC628	2.7f	1.67j	1.05ab	70.7a	89.2e	19.8k	1.92ef
SC645	3.1e	1.90cd	0.95de	68.5fgh	89.7de	20.6jk	1.83g
LSD	0.20	0.04	0.03	0.50	5.94	1.43	0.93

*Means with identical letters within each variable are not different (P< 0.05)

Table 5.5 Effect of growing location on the mean values of sorghum whole kernel attributes.

	Whole Kernel NIR				Single Kernel Analysis		
Location	**Fat**	**Fiber**	**Ash**	**Starch**	**Hardness**	**Wt**	**Dia**
Colby	3.7b*	1.80b	0.96b	68.5b	87.9b	23.9b	2.15b
Hays	3.8a	1.92a	0.91c	68.5b	89.4a	25.1a	2.21a
Manhattan	2.8c	1.72c	1.02a	69.7a	82.0c	25.0a	2.21a
LSD	0.08	0.02	0.01	0.20	1.48	0.57	0.04

*Means with identical letters within each variable are not different (P< 0.05)

Table 5.6 Effect of genotype on the mean values of sorghum starch and protein composition and functionality.

					Starch Granule Size Distribution						Protein Analysis	
Genotype	**Amylose**	**A Granule**	**B Granule**	**C Granule**	**<2µm**	**2-5µm**	**5-10µm**	**10-20µm**	**20-30µm**	**>30µm**	**Content**	**Digestibility**
B OK11	30.0abc*	44.0ef	45.2cd	10.8bc	4.82cdef	6.00bc	19.53bc	49.77fgh	14.70efg	5.18cdefg	12.60fgh	56.31bcdef
BTx Arg-1	19.2e	35.4h	51.9a	12.7a	5.66a	7.07a	23.72a	48.66hi	10.43j	4.46fg	12.02ghi	57.40bcde
BTx2752	26.0d	47.4bcde	42.4def	10.2bcde	4.40fghi	5.77bcde	17.63bcdef	48.99hi	17.78bcd	5.44bcdefg	11.15j	58.11abcd
BTx399	30.2abc	46.0de	43.9de	10.2bcde	4.96bcde	5.21fg	16.64fg	52.40c	15.74def	5.05defg	11.09j	57.24bcde
BTx643	31.3a	49.4bcd	40.8efg	9.9def	4.23ghi	5.64bcdef	16.35fg	49.40h	17.99bcd	6.40abc	12.38gh	53.35efg
P898012	29.0abcd	47.1bcde	42.0efg	10.9b	5.06bcd	5.85bcd	16.75fg	50.44defgh	16.97cde	4.92efg	12.84defg	60.38ab
RTx430	30.5abc	44.1ef	45.3cd	10.6bcde	4.52efghi	6.05b	19.66b	49.32h	15.72def	4.73efg	13.77bc	55.40cdef
SC1056	29.8abc	40.4fg	48.9ab	10.7bcd	5.29abc	5.41defg	17.84bcdef	54.58a	11.34ij	5.54bcdefg	13.36cdef	55.98cdef
SC108	27.2cd	41.0fg	48.9ab	10.1bcde	4.61defgh	5.51cdefg	19.47bc	51.24cdefg	12.18hij	6.99a	14.35ab	48.77hij
SC1104	29.1abcd	46.1de	43.6de	10.3bcde	4.45fghi	5.81bcde	16.64fg	51.81cde	15.86def	5.44bcdefg	13.63bcd	52.49fgh
SC1211	30.1abc	46.8cde	43.1de	10.1bcde	4.86cdef	5.28efg	16.55fg	52.50bc	16.25def	4.58efg	13.56bcde	54.38defg
SC1277	29.2abcd	50.7bc	39.4fg	9.9def	4.23ghi	5.66bcdef	15.23gh	49.57gh	18.99bc	6.31abcd	15.17a	47.45ij
SC38	29.0abcd	51.0b	39.2g	9.8ef	4.39fghi	5.41defg	16.95fg	47.29ij	20.12b	5.84abcde	11.90hij	59.17abc
SC391	29.3abcd	41.6fg	47.8bc	10.6bcde	5.01bcd	5.62bcdef	19.40bcd	51.46cdef	13.21ghi	5.29cdefg	12.74efg	53.90defg
SC414	29.5abcd	46.5de	43.1de	10.4bcde	4.64defg	5.73bcdef	17.36def	50.27efgh	16.45def	5.56bcdef	13.98bc	51.01ghi
SC471	31.4a	38.9gh	50.1ab	10.9b	5.44ab	5.47cdefg	19.12bcde	54.32ab	11.27ij	4.37g	12.52gh	62.05a
SC489	27.8bcd	57.1a	33.8h	9.1f	4.11i	5.03g	13.88h	45.66j	24.59a	6.73ab	11.4ij	59.71abc
SC628	30.7ab	47.7bcde	42.3defg	10.0cdef	4.14hi	5.86bcd	17.18efg	49.83fgh	17.09cd	5.90abcde	12.19ghi	55.85cdef
SC645	27.2cd	44.4ef	45.2cd	10.4bcde	4.75def	5.62bcdef	17.52cdef	52.22cd	14.11fgh	5.77abcdef	13.61bcd	45.58j
LSD	3.33	3.93	3.19	0.89	0.49	0.55	2.10	1.82	2.38	1.34	0.83	4.38

*Means with identical letters within each variable are not different (P< 0.05)

Table 5.7 Effect of growing location on the mean values of sorghum starch and protein composition and functionality.

		Starch Granule Size Distribution									Protein Analysis	
Location	**Amylose**	**A Granule**	**B Granule**	**C Granule**	**<2µm**	**2-5µm**	**5-10µm**	**10-20µm**	**20-30µm**	**>30µm**	**Content**	**Digestibility**
Colby	28.7b*	44.9a	44.7a	10.4a	4.67a	5.77a	17.77a	50.93a	15.81a	5.06b	12.98a	55.32b
Hays	26.3c	46.3a	43.4b	10.4a	4.70a	5.68a	17.73a	50.07b	16.23a	5.58ab	12.55b	57.38a
Manhattan	31.3a	45.5a	44.1ab	10.4a	4.77a	5.61a	17.77a	50.54ab	15.45a	5.86a	13.04a	52.22c
LSD	1.32	1.56	1.27	0.35	0.19	0.22	0.83	0.72	0.94	0.53	0.33	1.74

*Means with identical letters within each variable are not different (P< 0.05)

Table 5.8 Pearson correlation coefficients for sorghum attributes.

Factor	Amylose	A Granule	B Granule	C Granule	<2µm	2-5µm	5-10µm	10-20µm	20-30µm	>30µm	Fat	Fiber	Ash	Starch	Hardness	Weight	Diameter	Protein
Amylose	1.000																	
A Granule	ns*	1.000																
B Granule	ns	-0.996	1.000															
C Granule	ns	-0.836	0.784	1.000														
<2µm	-0.365	-0.711	0.695	0.675	1.000													
2-5µm	ns	-0.483	0.430	0.719	0.283	1.000												
5-10µm	-0.501	-0.854	0.832	0.826	0.621	0.686	1.000											
10-20µm	-0.302	-0.419	0.449	ns	0.595	ns	ns	1.000										
20-30µm	ns	0.879	-0.884	-0.681	-0.795	-0.391	-0.785	-0.671	1.000									
>30µm	ns	0.316	-0.292	-0.407	-0.582	-0.309	-0.284	-0.475	0.337	1.000								
Fat	-0.447	ns	ns	ns	ns	ns	ns	ns	ns	ns	1.000							
Fiber	-0.475	ns	ns	ns	ns	0.311	ns	ns	ns	ns	0.441	1.000						
Ash	0.405	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.886	-0.436	1.000					
Starch	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.464	-0.445	0.283	1.000				
Hardness	-0.422	ns	ns	ns	-0.272	ns	ns	ns	0.281	ns	0.414	0.411	-0.453	ns	1.000			
Weight	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.413	1.000		
Diameter	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.262	ns	-0.445	0.927	1.000	
Protein	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.294	-0.440	ns	ns	ns	1.000
Protein Digestibility	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.286	0.348	ns	-0.381	ns	ns	ns	ns	-0.628

*ns-not significant (P<0.05)

Chapter 6 - The effect of nitrogen fertilization and cover cropping systems on sorghum grain characteristics.

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Abstract

Cover crop treatments and nitrogen (N) fertilization rates were investigated for their impact on sorghum grain quality attributes. Sorghum was planted in field plots treated with differing cover cropping systems and fertilization rates. The size (weight and diameter) and hardness of the kernels were influenced by both the cover crop and N rates. The protein content increased as the N rate increased and also with the addition of cover crops to the system. The protein digestibility values and starch granule size distributions were not affected by N rate or the cover cropping treatments. Soil properties were tested to determine relationships with grain quality attributes. The utilization of cover crops appears to increase the protein content without causing a deleterious effect on protein digestibility. The end-product quality is not hampered by the use of beneficial cropping systems necessary for sustainable agriculture.

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is the 5th leading cereal grain produced worldwide. Because sorghum is tolerant to heat and drought conditions, it is commonly grown under non-irrigated conditions in semi-arid parts of the United States, such as Kansas, Oklahoma, and Texas. In 2003, 25% of U.S. sorghum acreage was grown under a cultural practice known as no tillage, with estimates of 34% utilization of no tillage by 2009 or an annual increase of 1.5%[1]. No tillage systems typically use herbicides rather than mechanical cultivation for weed control and seedbed preparation, thus providing benefits to the soil by reducing erosion and increasing soil organic matter content.

Recently, cover crops have been added to no till cropping systems. Cover crops do not produce a marketable product, but they have many benefits, including: increasing organic matter content, providing residue cover, preventing or reducing soil erosion, cycling nutrients, reducing nitrate leaching, suppressing weeds, and adding diversity to crop sequences[2].

Nitrogen (N) fertilization effect on grain quality has been studied extensively in many cereal grains. Numerous studies in wheat have shown that increasing N levels leads to an increase in wheat protein content[3-5]. Similar effects have been found in triticale[6] and maize[5]. In addition to fertilization, crop rotation or cropping systems also affect the grain quality in wheat. Galantini et al.[7] found that wheat grown in a rotation with a legume was higher in protein content as well as higher yielding than wheat grown in a rotation with another grass. The effect of tillage has also been investigated for impact on the grain quality. No tillage systems produced wheat with lower protein content than conventional tillage systems[8]. However, long term studies have shown that this effect was caused by an increase in N immobilization and can be alleviated with increased N fertilization rates[5].

Prior work on crop rotation and soil treatment effects on sorghum has shown grain test weight, hardness and protein content increases with increases in available soil N. Kaye et al.[9] investigated different sources of N on a conventionally tilled soil with differing preceding crops grown on specific plots. Virtually no research has been conducted to determine how N rates as well as tillage practices impact sorghum grain composition and quality. Therefore, the objectives of this study were (1) to determine the effect of cover crops and N fertilization on the physical grain characteristics of sorghum, and (2) to determine the protein and starch quality of sorghum grown under different cropping systems.

Materials and Methods

Cover Crop System

The grain samples were obtained from a long-term cover crop experiment located near Hesston, KS (38°8'24'' N, 97°25'48'' W). The soil is a Geary silt loam (fine-silty, mixed, superactive, mesic Udic Argiustoll) with < 3% slope and is deep and moderately well-drained. The study region receives 874 mm of annual precipitation and has a mean annual temperature is 14.4°C. The study was initiated in 1995 with a winter wheat [*Triticum aestivum* (L.)] and grain sorghum [*Sorghum bicolor* (L.)] crop rotation. From 1995 to 2000 hairy vetch (*Vicia villosa* Roth) was planted as a winter cover crop between the wheat and sorghum crops and the whole site was managed with reduced tillage. From 2000 to 2002, no cover crop was planted and the entire site was planted to winter wheat. From 2002 to 2009, the cover crop treatments were none, late soybean (*Glycine max* L.), or Sunn hemp (*Crotalaria juncea* L.) under no-till management. The experimental design was randomized complete block with a factorial design. There were three cover crop treatments (none, late soybean, and Sunn hemp) and four N rates (0, 33, 66, and 100 kg N/ ha), replicated four times, for a total of 48 plots. The cover crops were planted during the summer after wheat crop was harvested, and terminated in early autumn. In 2009, sorghum grain samples were harvested from the center two rows using a mechanical plot combine. The plot size was 6 by 13.5 m. For a detailed description of complete field operations during the history of the experiment, refer to two previously published papers by Blanco-Canqui et al.[10,11] on the effects of the management practices on soil properties and crop yields, respectively.

Soil Testing

Soil samples were collected from 0-7.5-cm of each plot in early spring 2010 from the center, non-trafficked row of each plot[11]. The samples were air dried and ground to pass through a 250-µm sieve to determine the total organic C and N concentration by the dry combustion method of Nelson and Sommers[12].

Grain Processing

A sample of grain from each of the 48 field plots was used for chemical analysis. An UDY mill (Udy Corporation, Fort Collins, CO) equipped with a 0.5 mm screen was used to produce whole grain meal used in protein analysis techniques. For starch isolation, grain samples were first decorticated (20% removal) using a TADD (Venables Machine Works) and then decorticated grain meal was produced similarly to the whole grain meal.

Grain Hardness and Sizing

The physical attributes of the sorghum kernels (hardness, diameter, and weight) were measured using a single kernel characterization system (SKCS 4100, Perten Instruments) controlled by SKCS for Windows software (Version 2.1.0.1) using 100 kernels per sample[13].

Chemical Analysis of Sorghum Meal

Total protein content of the whole grain was determined using a N combustion method (AACC method 46-30)[14] using a LECO FP-528 Nitrogen Determinator (St. Joseph, MI). Nitrogen values were converted to protein by multiplying by 6.25. Protein digestibility was determined using the modified pepsin method described by Mertz et al.[15] with the residues analyzed by N combustion.

Kafirins were extracted for analysis as described in Bean et al.[16] The extracted kafirins were analyzed via reversed phase high performance liquid chromatography (RP-HPLC) as described in Bean et al.[16] using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA) equipped with a Poroshell 300SB-C8 column.

The Megazyme Total Starch Assay kit (K-TSTA, Megazyme International, Wicklow Ireland) with the DMSO pretreatment (AACC Method 76-13)[17] was used to determine the total starch content of the whole milled sorghum. Starch content was corrected to a dry basis content. Starch was isolated from the decorticated sorghum meal by the sonication method of Park et al.[18]

Starch granule size distributions were measured using a single wavelength Beckman Coulter LS 13 320 Particle Size Analyzer (Miami, FL) with the Universal Liquid Module (ULM) for liquid-based measurements. Data were calculated as volume percent measurements and binned according to common size groupings: A-type (>15μm), B-type (5-15μm), and C-type (<5μm).

Whole grain sorghum meal samples were analyzed for mineral concentration by Ward Laboratories (Kearney, NE). Samples were analyzed for Ca, P, K, Mg, Zn, Fe, Mn, Cu, S, and Na concentrations.

Results and Discussion

Soil Properties

Soil organic C and total N concentration in the 0-7.5-cm soil depth were both significantly affected by the long term N rate and cover crop treatments (Table 6.1). The mean values for SOC and total N gradually increased with higher N rates. The late soybean and Sunn hemp treatments had statistically similar SOC and total N levels, and were significantly greater than the plots where cover crops were not grown. An extensive evaluation of soil properties was reported for the same experiment by Blanco-Canqui et al.[11]. Organic matter is an extremely complex substance, and is largely comprised of SOC and total N. Therefore, increasing SOC and total N gradually over a period of several years has led to an increase in soil organic matter, which is considered a critical part of building healthy soils and is the foundation of sustainable agriculture[19]. It is important to remember that the study was established in 1995, and the soil samples were collected in early spring 2010, thus these changes in SOC and total N are a cumulative effect of many years of management.

Whole Kernel Properties

The effect of N rate on the single kernel measurements is reported in Table 6.2. The hardness values appear to increase as the level of N increases across all cover crop treatments. The resultant hardness of the grain from N applications of 0kg/ha and 33kg/ha is significantly lower than from 66kg/ha and 100kg/ha of N applications. The utilization of a cover crop system also increased the SKCS hardness value. While there was not a significant difference in hardness of the grain between the late soybean (74.7) and Sunn hemp (74.6) cover crop system. Hardness index was higher in in plots with than without cover crops.

The kernel size also significantly responded to both the N fertilization and cover crop systems. The 0 and 100kg/ha rate had kernels of similar diameter and weight. The kernel size for the two intermediate N levels (33 and 66kg/ha) was larger and heavier than the 0 and 100kg/ ha levels. Batey and Reynish[3] observed a reduction in grain size of wheat grown on increasing

levels of N fertilization. They hypothesized that the decline in grain size was due to additional tiller survival, therefore more kernels per plant to divide the carbohydrate produced through photosynthesis. The significantly higher number of heads/plant observed in the 100kg/ha treatment versus the lower N rates could explain the reduction in kernel size of the 100kg/ha treatment. Cover crop plots produced kernels that were significantly heavier and larger than plots without cover crop. The cover cropped plots had kernels that were numerically similar to that of the intermediate N rates for both kernel diameter and weight. Cover crop and intermediate N levels exhibited a range in heads/plant from 1.33 to 1.40, suggesting that this range is the optimum value for heads/plant to maximize the kernel size.

Protein Properties

The protein content across increased with higher rates of N fertilization, from 8.1% protein (0kg/ha) to 9.1% protein (100kg/ha) (Table 5.3). The 33kg/ha rate (8.5% protein) did not differ from 0kg/ha rate, but the 66kg/ha rate (8.9%) was different from both the 0 and 100kg/ha rates. This result is in agreement with prior research that showed that protein of cereal grains is related to the N fertilization level. Batey and Reynish[3] demonstrated that increasing N fertilization rates led to an increase in grain protein content in wheat. In triticale, Lestingi et al.[6] found not only that increasing N fertilization increased grain protein content, but also that tillage systems affected grain quality parameter including protein content. The cover cropped plots produced sorghum with a higher protein content than that of the non-cover cropped control. The plots cover cropped with soybean had the highest protein content at 9.2%, followed by the Sunn hemp plots that produced grain with 8.8% protein while the control had a protein content of 8.2%. Galatini et al.[7] showed that wheat grown in a wheat-legume rotation exhibited an increase in protein content in the grain as well as an increase in production.

RP-HPLC was used to quantify kafirin subclass composition of the samples. The 100kg/ha treatment exhibited a greater peak area for the γ-kafirins than the other fertilizer levels, however the proportion of γ-kafirins peak area to total peak area exhibited no differences among N levels. A similar result was found with the cover crop treatments. The plots utilizing soybean as a cover crop had a greater γ-kafirin peak area than the plots without a cover crop. The relative percentage of γ-kafirin did not statistically differ across the three cover crops.

Since the primary utilization of sorghum in the United States is for animal feed, the digestibility of sorghum proteins can be an important end-use quality trait. The N and cover crop treatments did not display any significant difference in the digestibility (Table 6.3). The utilization of cover crops or various N fertilization levels does not create any deleterious effect on the proportion of protein that is digestible.

Starch Properties

The total starch content of the whole milled sorghum did not show any effect of N fertilization levels. The 0 kg/ha fertilization level had a total starch content of 75.3% whereas the 100/kg/ha level had a total starch content of 73.9%. The intermediate N levels had total starch contents in between the aforementioned values; however, there were no significant differences among the fertilization levels. The plots with Sunn hemp as a cover crop were significantly higher in total starch content than the plots with soybean. The non-cover crop treatments were not significantly different from the two cover crop treatments with total starch content of 74.9%. In addition to the total amount of starch present, the granular architecture (size distribution) of the starches is also related to its functionality.

Starch granules are commonly organized into three size types (A, B, and C). The A-type granules (>15µm) make up the largest proportion of the total volume of starch, followed by the B-type granules (5-15µm). The C-type (<5µm) have the smallest proportion of volume, but typically outnumber the other types numerically. The ratio of granule types can be found in Table 4. In the A-type granules only the 100kg/ha N rate produced lower proportion statistically than 0 and 33kg/ha levels. The 66kg/ha was not statistically separated from neither higher nor lower N treatment levels. Conversely, the highest N level produced the highest proportion of B-type granules. The C-type experienced no differences across the N treatments. The starch granule size distribution was not affected by the cover cropping systems. There were no statistical separations in any size grouping (Table 6.4). Since there were very minimal differences in the total content and the granule size distributions, the functionality of the starch would most likely not be affected by the cropping systems.

Mineral Analysis

The mineral concentrations of the sorghum meal were not affected by the N treatments except for phosphorus (P) and sulfur (S) (Table 6.5). The concentration of P decreased as the

level of N fertilizer increased. The decrease in P is similar to that found in a study by Zebarth et al.[20] on wheat. The S concentration exhibited the largest differences due to treatments for both the N fertilization rate and the cover crop. The S concentration increased 9.3% from the 0kg/ha (0.0917% S) to 100kg/ha (0.1033% S) treatments. A similar increase was seen in the cover crop treatments with soybean (0.1038% S) having the highest concentration followed by the Sunn hemp (0.0981% S) and the no cover crop (0.0931% S) plot the lowest concentration. Sulfur fertilization studies in wheat have shown that as the S concentration increases in the grain, the composition of the grain proteins changed, thus affecting the flour's functionality in dough mixing[4]. However, the increasing S concentration resulting from the fertilization and cover cropping did not appear to alter the digestibility of the proteins. A future study is needed to determine the mechanism for the increasing S concentration.

Relationships with soil properties

The soil properties that were studied showed some significant correlations to both physical and biochemical grain characteristics. Correlations can be seen in Table 6.6. Grain hardness was positively correlated with both the total soil N ($r = 0.476$) and soil organic carbon ($r = 0.509$) tests. The protein content and composition was also positively correlated with the soil properties, thus improvements made in soil quality will enhance the grain properties. The total starch content was not correlated to the soil parameters, but the granule size distribution was related to the soil organic carbon content. A-type granules were negatively correlated ($r = -0.288$) whereas B-type granules were positively correlated ($r = 0.292$). The overall functionality of the sorghum starch appears not to be as affected by soil properties as do the protein components.

In summary, the N fertilization and cover cropping systems appeared to enhance the soil fertility by increasing both total soil N and soil organic carbon. The cover crop systems provided an increase in the agronomic effect as well as overall sustainability of the production system without causing deleterious effects on the end product quality. Physical grain characteristics were influenced by the N rates and the utilization of a cover crop. The treatments also increased the amount of protein in the grain without reducing any digestibility, thus allowing for a greater digestible protein yield. The type of cover crop used also exhibited slight differences; therefore more research is needed to examine this finding. Since, cover cropping appears to provide both

agronomic and end product quality benefits increased utilization of this cropping system could be useful for developing sustainable agricultural systems.

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Table 6.1 Soil properties after a long term cover crop system and N fertilization rate study.

	Soil Organic Carbon g/kg	Total N g/kg
N Rate		
0	13.92c*	1.37b
33	15.61bc	1.5ab
66	17.25ab	1.69a
100	18.18a	1.61a
LSD	1.75	0.21
Cover Crop		
None	13.66b	1.32b
Late Soybean	16.9a	1.63a
Sunn Hemp	18.16a	1.67a
LSD	1.52	0.18

*Means with identical letters within each variable and study factor are not different ($P < 0.05$)
Data was adapted from Blanco et al.[11]

Table 6.2 Physical characteristics of sorghum kernels grown under differing N fertilization rates and cover crop systems.

	Hardness unit	Weight mg	Diameter mm	Test Weight kg/(m^3)	Grain Yield kg/ha
N Rate					
0	68.7b*	23.6b	2.16b	687.3a	5110.9b
33	70.0b	24.0a	2.21a	714.3a	6609.6b
66	76.2a	24.4a	2.21a	720.7a	7763.5a
100	77.3a	23.3b	2.18b	727.2a	7964.2a
LSD	3.1	0.6	0.03		464.1
Cover Crop					
None	69.7b	23.4b	2.16b	713.0a	6302.4c
Late					
Soybean	74.7a	23.9a	2.21a	705.3b	6741.3b
Sunn Hemp	74.6a	24.0a	2.21a	718.1a	7537.7a
LSD	2.7	0.5	0.03	6.4	376.3

*Means with identical letters within each variable and study factor are not different (P< 0.05)

Table 6.3 Protein analysis of sorghum grown under differing N fertilization rates and cover crop systems.

	Protein Content % D.B.	Protein Digestibility %	γ-kafirin PA[a] mAU	Non γ-Kafirin PA mAU	Total PA mAU	γ-kafirin PA/PC[b]	γ-kafirin % TPA
N Rate							
0	8.1c[*]	73.6a	3810b	52268c	56078c	472a	6.82a
33	8.5bc	74.5a	3917b	55197bc	59114bc	461ab	6.65a
66	8.9b	73.3a	3948b	56063b	60011b	441b	6.57a
100	9.5a	73.0a	4323a	61844a	66168a	456ab	6.55a
LSD	0.45	1.88	271	3510	3718	28	0.3
Cover Crop							
None	8.2c	74.2a	3811b	52956c	56767c	464a	6.75a
Late							
Soybean	9.2a	73.4a	4171a	59823a	63994a	452a	6.53a
Sunn Hemp	8.8b	73.2a	4017ab	56250b	60267b	457a	6.67a
LSD	0.39	1.6	235	3040	3220	24	0.26

[a] PA-peak area
[b]PC-protein content
[*]Means with identical letters within each variable and study factor are not different (P< 0.05)

Table 6.4 Starch properties of sorghum grown under differing N fertilization rates and cover crop systems.

	Total Starch % D.B.	A-Type Granules Volume %	B-Type Granules Volume %	C-Type Granules Volume %
N Rate				
0	75.3a*	52.3a	38.9b	8.8a
33	74.2a	52.6a	38.6b	8.8a
66	74.7a	50.4ab	40.4ab	9.3a
100	73.9a	48.7b	41.9a	9.4a
LSD	1.92	3.31	2.62	0.72
Cover Crop				
None	74.9ab	50.4a	40.3a	9.3a
Late Soybean	73.4b	52.1a	39.1a	8.7a
Sunn Hemp	75.2a	50.4a	40.3a	9.3a
LSD	1.66	2.87	2.27	0.62

*Means with identical letters within each variable and study factor are not different ($P < 0.05$)

Table 6.5 Mineral concentrations of sorghum grown under differing N fertilization rates and cover crop systems.

	Ca	P	K	Mg	Zn	Fe	Mn	Cu	S	Na
	%	%	%	%	ppm	ppm	ppm	ppm	%	%
N Rate										
0	0.0367a*	0.3625a	0.4658a	0.1542a	16.28a	89.08a	13.00a	2.73a	0.0917c	0.0258ab
33	0.0375a	0.3658a	0.4700a	0.1567a	16.96a	99.08a	14.17a	2.67a	0.0975b	0.0292a
66	0.0367a	0.3575ab	0.4641a	0.1550a	16.33a	97.58a	13.50a	2.89a	0.1008ab	0.0250b
100	0.0333a	0.3442b	0.4567a	0.1542a	18.00a	75.75a	13.42a	2.83a	0.1033a	0.0267ab
LSD	0.0079	0.0179	0.0221	0.0074	2.19	40.13	2.77	0.37	0.0048	0.0040
Cover Crop										
None	0.0388a	0.3594a	0.4681a	0.1519a	16.19a	83.94a	13.31a	2.63a	0.0931c	0.0269a
Late Soybean	0.0356a	0.3550a	0.4606a	0.1550a	17.24a	99.31a	13.63a	2.95a	0.1038a	0.0263a
Sunn Hemp	0.0338a	0.3581a	0.4638a	0.1581a	17.24a	87.88a	13.63a	2.76a	0.0981b	0.0269a
LSD	0.0068	0.0155	0.0191	0.0064	1.90	34.76	2.40	0.32	0.0041	0.0035

*Means with identical letters within each variable and study factor are not different ($P < 0.05$)

Table 6.6 Pearson correlation analysis of soil properties to sorghum kernel characteristics.

	Hardness	Diameter	Wt.	Protein	Digestibility	γ-kafirin PA	Non γ-kafirin PA	Total PA	A-Granules	B-Granules	C-Granules
Total Soil Nitrogen	0.48	ns*	ns	0.40	ns	0.32	0.38	0.38	ns	ns	ns
Soil Organic Carbon	0.51	ns	ns	0.48	ns	0.40	0.43	0.44	-0.29	0.29	ns

*ns-not significant ($P<0.05$)

Chapter 7 - Summary

Hypothesis 1: Sorghum grain chemistry will be altered due to kernel development.

This hypothesis was found to be true. The starch granule size distribution, amylose content, crystallinity, and protein content were altered due to kernel DAA.

Hypothesis 2: Sorghum starch and protein end-use qualities will be altered due to kernel development.

The thermal properties of the starch were very dependent on the kernel maturity. Starch and protein digestibility was also found to be dependent on the stage of maturity.

Hypothesis 3: Sorghum grain properties and protein digestibility are affected by both genetic and environmental factors.

The physical properties of the sorghum were greatly affected by both the genotype and the environment. However the starch granule size distribution was only affected by the genotype. The protein digestibility was also variable due to genotypic and environmental factors.

Hypothesis 4: Agronomic practices can affect the grain chemical and physical properties of sorghum.

The physical properties of sorghum kernel such as hardness and size are enhanced by the utilization of a cover cropping system. The protein content was increased with the cover crops as well as increasing nitrogen fertilization rates, however the digestibility of the protein was unaffected.