Isolation, structure, and functional properties of waxy sorghum starches and flours

by

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Abstract

Waxy sorghum contains essentially only amylopectin in its endosperm starch and has unique properties that could be potentially used as value-added food ingredients and increase its value compared with normal sorghum. Currently, waxy maize starch is the preferred base starch, which is further chemically modified to produce food thickeners. However, clean label is an emerging global trend in packaged foods, and waxy sorghum flours and starches have the potential to be directly used as effective thickeners in food applications. In this study, a comprehensive investigation was conducted to evaluate the potential of waxy sorghum starches and flours as food thickeners by examining their structural and functional properties in comparison to waxy maize starch.

The objectives of the first part of the study were to develop an efficient process for isolating starch from decorticated and whole waxy sorghum grains using alkaline protease, and to compare the properties of starches isolated by enzymatic with that of starches isolated from traditional wet milling methods. To evaluate the efficiency and effectiveness of the isolation process, key parameters such as starch recovery, yield, residual protein, ash, damaged starch content, color, as well as pasting and thermal properties were determined. Starch recovery showed no statistically significant differences among the three methods, and all isolated starches exhibited desired purity regardless of the method employed. However, enzymatic treatment methods not only produced starches with lower residual protein (0.18-0.22%) but also resulted in significantly greater peak (1992-2023 cP), breakdown (1123-1172 cP), and setback viscosities (275-355 cP) in comparison to the wet milling. The starches isolated by wet milling and enzymatic treatment of decorticated sorghum exhibited similar onset and peak gelatinization temperatures, while both methods produced starches with better color quality

(higher L*, lower b*) compared to the enzymatic treatment of whole sorghum. Overall, the enzymatic treatment of decorticated sorghum could be used as an effective method for starch isolation at the laboratory scale, with promising potential for application in industrial-scale processes.

The objectives of the second part of the study were to identify waxy sorghum starches and flours with greater thickening power and cold storage stability, determine the relationship between pasting properties and morphology of waxy starch during cooking, understand how the retrogradation properties are affected by chain-length distribution, and investigate correlations between the starch content, starch structural features, and functional properties of waxy sorghum starches and flours. Waxy sorghum starches exhibited a greater degree of granule swelling and significantly higher pasting viscosities compared to waxy maize starch. Starches with lower retrogradation tendency contained a higher proportion of chains with DP 6-11 and a lower proportion of chains with DP 12-24. The peak, setback, and final viscosities of waxy sorghum flours were significantly positively correlated with the corresponding viscosities of their starches (peak: $r = 0.606^*$; setback: $r = 0.635^*$; final: $r = 0.656^*$, $p < 0.05$). The total starch content was only found to be significantly correlated with setback ($r=0.694^*$) and final viscosities ($r=0.682^*$). In contrast, grain hardness exhibited a significant negative correlation with the peak ($r =-0.785^*$) and breakdown ($r =-0.780^{**}$, $p<0.01$) viscosities of waxy sorghum flours.

The third part of the study was conducted to investigate the impact of protein matrix on pasting properties of waxy sorghum flours. The objectives were to obtain waxy sorghum flours with different protein levels using enzymatic treatment or solvent extraction methods and to investigate how the protein matrix affects the pasting properties after cooking by using a

confocal laser scanning microscope (CLSM). Protease treatment was an effective way to hydrolyze and remove part of the protein and obtain flours with reduced protein contents. Unmodified waxy sorghum flours with a higher protein content exhibited lower pasting peak viscosity due to the protein matrix inhibiting starch swelling by forming a physical network around starch granules and restricting the swelling of starch granules during pasting. The hydrolysis of the protein matrix resulted in increased peak pasting viscosities by disrupting the protein network that restricted the swelling of starch granules. These findings show the critical role of the proteins in modulating the functional properties of waxy sorghum flours, providing valuable insights for optimizing their use in food applications, such as effective thickeners and gluten-free products.

Table of Contents

List of Figures

List of Tables

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Chapter 1 - Introduction

General background

Sorghum (*Sorghum bicolor* L. Moench) is the 5th most widely grown cereal crop worldwide, produced behind maize, rice, wheat, and barley (Bean et al., 2019; Zhu, 2014). The United States is the largest producer of sorghum, accounting for over 14% of global sorghum production, followed by Nigeria, India, Mexico, Brazil (U.S. Department of Agriculture, 2024). Due to its low production costs, drought resistance, and tolerance to high temperatures, sorghum plays a critical role in food security in some semiarid areas of Asia, Africa, and South America (Bean et al., 2019; El Halal et al., 2019; Sang et al., 2008; Shaikh et al., 2024). However, it is still an underutilized crop in most developed countries, with sorghum being mainly used for animal feed, bioethanol production, and industrial purposes (Ai et al., 2011; Bean et al., 2019; Ramatoulaye et al., 2016; Wu et al., 2013; Yan et al., 2011).

Sorghum is an important food source for future human consumption due to its distinct nutritional characteristics and health benefits, such as high dietary fiber, antioxidants, anticancer, and anti-inflammatory (Abah et al., 2020; Awika & Rooney, 2004; Espitia-Hernández et al., 2022; Lee et al., 2020). As a gluten-free grain, sorghum also serves as a promising alternative flour for individuals with celiac disease and gluten intolerance (Ciacci et al., 2007; Pineli et al., 2015; Pontieri & Del Giudice, 2016). As a result, sorghum has been increasingly utilized in a wide range of food products, such as fermented beverages, flatbreads, noodles, cookies, tortillas, and porridges (Khoddami et al., 2023; Pineli et al., 2015; Pontieri & Del Giudice, 2016; Taylor & Anyango, 2011).

Starch, the major storage carbohydrate in sorghum grains, constitutes up to 80% of the dry grain weight (Liu et al., 2012; Zhu, 2014), which is an important source for industrial and domestic uses (Olayinka et al., 2013). The utilization and quality of sorghum-based products are largely influenced by the properties of its starch. Many important physicochemical and nutritional properties of starch are affected by the ratio of amylose and amylopectin, the two major polymers in the starch granules, and the structure of amylopectin (Bean et al., 2019; Hsieh et al., 2019; Sang et al., 2008). Most of the sorghum genotypes contain amylose at around 20% to 30%, which is typical for normal cereal grains (Zhu, 2014). However, waxy sorghum contains essentially only amylopectin in its endosperm starch and has unique starch properties that could be used as value-added food ingredients, potentially enhancing its economic value compared to normal sorghum (Hsieh et al., 2019). In general, amylose prompts the gelling and film-forming properties of starch, whereas amylopectin, on the other hand, increases the thickening power of starch and promotes the clarity of starch pastes and gels, which are crucial for controlling the consistencies of soups, gravies, sauces, and fillings (Sang et al., 2008). This chapter will focus on the chemistry and composition of major components (starch and protein), current uses and prospects of waxy sorghum grains.

Structure and chemical characteristics of sorghum starch and protein

Sorghum starch is synthesized and deposited in the endosperm, providing the energy source for reproduction and development (Bean et al., 2019). Sorghum starch consists of two types of glucose polymers, amylose and amylopectin. Amylose is an almost entirely linear form of α-1,4 glucan, whereas amylopectin is a highly branched form of α-1,4 glucan with α-1,6 branch points (James et al., 2003; Kawahigashi et al., 2013). The ratio of amylose to amylopectin in starch granules of sorghum grain is critical for the texture and appearance of the endosperm

(Bean et al., 2019). Normal sorghum starches contain 20% to 30% amylose, whereas waxy sorghum starches contain very low or nearly undetectable levels of amylose (Zhu, 2014). Sang et al. (2008) compared the structure and physicochemical properties of sorghum starches with different amylose levels. The results showed that starch gelatinization and retrogradation, pasting properties, and rheological properties are strongly affected by amylose content. The waxy sorghum grains with low or no amylose content have been shown to have higher ethanol conversion rates and increased starch and protein digestibility compared with wild-type grain (Ai et al., 2011; Wang et al., 2008; Wong et al., 2009; Yangcheng et al., 2013), indicating potential applications for waxy sorghum in the food and bioenergy industries. In addition, using waxy sorghums for ethanol production has multiple advantages, including higher free amino nitrogen, higher starch and protein digestibility, easier gelatinization, low viscosity during liquefaction, and shorter fermentation times (Yan et al., 2011). Therefore, waxy is one of the most important traits in breeding programs for improving sorghum cultivars. Further study on the structure of sorghum starch is of importance in expanding its application.

Biosynthesis of waxy sorghum starch

Starch is biosynthesized by the coordinated enzymatic actions of ADP-glucose pyrophosphorylase (AGPase), starch synthases (SS), starch-branching enzymes (SBE), starch-debranching enzymes (DBE), phosphorylase (Pho), and glucan water dikinase (GWD) (Blennow et al., 2013). Each enzyme may exist in two or multiple isoforms. Starch composition (e.g. ratio of amylose to amylopectin) and structure may be altered during biosynthesis to change the functional and nutritional properties of starch. Key enzymes responsible for sorghum starch biosynthesis have been identified, with their structure and properties reported by Xiao et al. (2022a).

The development of waxy starches has been achieved by two approaches: transformation by genetic modification (GM) and spontaneous mutation (non-GM). In recent years, the demand for clean-label ingredients has become of primary concern, and that concern discourages the food-use of GM ingredients. Thus, researchers have focused on the development of a non-GM waxy sorghum starch. The biosynthesis of amylose in cereal endosperm requires the granule-bound starch synthase (GBSS) (Denyer et al., 2001). Loss-of-function *gbss* mutants contain very low or undetectable levels of amylose and mostly amylopectin, resulting in the waxy endosperm phenotype (Ainsworth et al., 1993; Denyer et al., 2001; Shure et al., 1983; Taira et al., 1995; Wang et al., 1995). Thus, GBSS is frequently referred to as "waxy" protein.

Two types of waxy mutants, wx^a and wx^b, were previously identified in sorghum by PCR-based markers (Sattler et al., 2009). The study found that amylose levels in the starch of wx^a and wx^b lines were as low as 1.1% to 1.8%, compared to about 23% in non-waxy sorghum. The wx^a allele is associated with a large insertion in the third exon of the SbGBSS gene, resulting in the absence of the SbGBSS protein and, consequently, a waxy phenotype. The wx^b allele involves a missense mutation in the SbGBSS gene, resulting in lower SbGBSS enzyme activity and an altered endosperm with minimal amylose (Sattler et al., 2009). The mutations in the wx^a and wx^b alleles lead to starch with drastically reduced amylose content. Later, Kawahigashi et al. (2013) identified a novel waxy allele wx^c, found in a Taiwanese landrace, which was caused by a point mutation at the splice site, leading to suppression of GBSS I expression. Consequently, no GBSS I protein was produced, similar to wx^a, and this mutation led to a significant reduction of amylose content, as shown by the very low amylose content (0.71%) in the wx^c accessions.

Isolation and composition of waxy sorghum starch

To enhance the utilization of sorghum starch in food, feed, and industrial applications, an efficient and cost-effective isolation process should be developed to overcome challenges posed by its interaction with highly cross-linked kafirin proteins, the presence of polyphenols, and the specific grain structure (Eckhoff & Watson, 2009; El Halal et al., 2019). The wet milling process is commonly used for sorghum starch production on an industrial scale (Eckhoff & Watson, 2009), involving grain cleaning, sulfur dioxide steeping, grinding, separating components with hydrocyclones, and purifying and drying the starch. A variety of methods have been investigated for the isolation of sorghum starch at the laboratory scale, including the application of alkali solutions, sulfite treatments, lactic acid, and hydrolytic enzymes, individually or in combination, to separate starch from proteins, fibers, and lipids (Ahmed et al., 2016; Boudries et al., 2009; Eckhoff & Watson, 2009; El Halal et al., 2019; Higiro et al., 2003; Micaela & Drago, 2020; Pérez Sira & Lares Amaiz, 2004; Beta et al., 2000; Wang et al., 2000; Xie & Seib, 2000).

In a conventional wet milling process, grain sorghum was steeped in water containing sulfur dioxide (SO_2) as a reducing agent and lactic acid for 24 to 48 hours (Buffo et al., 1998; Cruz et al., 2015; Higiro et al., 2003; Xie & Seib, 2000). The addition of lactic acid significantly increased starch yield compared to sulfur dioxide (SO2) alone (Pérez et al., 2001) while also controlling microbial growth by reducing the pH of the steeping solution (Wang et al., 2000). However, the effective separation of sorghum starch is challenging due to the presence of polyphenols like tannins in some varieties that give off color to the resultant starch (Zhu, 2014). Some studies reported that alkaline treatments (NaOH) effectively improved starch brightness and whiteness by reducing phenolic compounds (Belhadi et al., 2013; Beta et al., 2001; Pérez

Sira & Lares Amaiz, 2004; Yang & Seib, 1996), while decortication prior to wet milling could also reduce pigmentation by removing the outer layers of sorghum grains (Higiro et al., 2003).

Pérez Sira & Lares Amaiz (2004) explored the use of alkaline reagents, including a mixture of sodium hypochlorite and potassium hydroxide, to bleach starch and effectively remove phenolic compounds responsible for discoloration. This method may raise environmental and safety concerns due to the use of chemicals like sodium hypochlorite and potential residues from the bleaching process. High-intensity ultrasound offered a rapid approach with minimal chemical use, producing high-purity starch from sorghum and other cereal grains (Park et al., 2006; Zhao et al., 2008). In addition, protease enzymes have been applied in the isolation of cereal starch to achieve higher-purity starch with lower residual protein content (Chew-Guevara et al., 2016; Dorglamud et al., 2013; Sittipod & Shi, 2016; Tester et al., 2007, 2008; Zhu et al., 2010). Although the starch and protein matrix in sorghum interact strongly, protease treatment has been shown to effectively hydrolyze protein and release starch granules (De Mesa-Stonestreet et al., 2010; Mezo-Villanueva & Serna-Saldívar, 2004). Chew-Guevara et al. (2016) reported that the combination of decortication and neutral protease (Neutrase®) treatment significantly increased the starch yield from 71.9% to 91.8% and decreased the residual protein from 0.51% to 0.12%, compared to isolation process without these treatments. It should be noted that variations in isolation methods and conditions may result in notable differences in the composition and functional properties of starch (Perez et al., 1997; Beta et al., 2001), thus offering the possibility of controlling starch functionalities.

Starch granular shape, size and crystallinity

Sorghum starch granules are mostly spherical and polygonal, with indentations and pores on the surface (Ai et al., 2011; Sang et al., 2008). Environmental factors, such as temperature,

have been shown to significantly reduce the diameter of granules due to increased heat exposure (Li et al., 2013). Waxy sorghum starch granules typically range from 4 to 20μm in diameter (Yang et al., 2019), while heterowaxy and normal sorghum starches exhibit similar diameter, ranging from 5 to 20μm (Sang et al., 2008). It should be noted that starch granules from the vitreous endosperm are more compact than those from the floury endosperm of sorghum (Ai et al., 2011; Yang et al., 2024). The waxy endosperm exhibited a looser structure with a greater number of spindle-shaped holes on the starch surface (Yang et al., 2024). Starch possesses a partially crystalline structure primarily attributed to amylopectin, which forms A-, B-, and C-type crystalline polymorphs. These structural characteristics are commonly analyzed using X-ray diffraction (XRD) (Zhu, 2014). The sorghum starch showed A-type X-ray polymorphs with strong diffraction peaks predominantly observed at $2\theta = 15°,17°,18°$, and 23° (Ai et al., 2011; Olayinka et al., 2013; Yan et al., 2023). The relative crystallinity of various sorghum genotypes has been reported to range from 20.5% to 32.4% (Yan et al., 2023) and 25.8% to 29.6% (Ai et al., 2011). These crystallinity levels are strongly influenced by apparent amylose content (AAC), exhibiting a significant negative correlation (Pérez & Bertoft, 2010). For example, a waxy sorghum with 4.8% amylose showed a crystallinity of 50.5%, while sorghum starches with low amylose (6.4% and 8.3%) demonstrated crystallinities of 28.7% and 22.1%, respectively (Kang et al., 2023; Yan et al., 2023).

Structural characteristics of amylopectin

Waxy sorghum contains negligible amylose and almost all amylopectin molecules in its endosperm starch. Amylose is a mostly linear polymer of α-1,4 linked D-glucose with a few α-1,6 branch points (Bean et al., 2019). However, amylopectin is the major component of starch and is larger than amylose, which is composed of a highly branched polymer consisting of α-1,4

linked D-glucose units with branches linked by α-1,6 bonds (Bean et al., 2019; Zhu, 2014). The molecular weight (Mw) of amylopectin is typically characterized by its weight-average Mw, which can be measured using size exclusion chromatography (SEC) (Zhu, 2014). Some studies reported that the molecular weight (Mw) of amylopectin in waxy sorghum varieties ranged from 8.4×10^7 to 9.9×10^7 g/mol at different growth stages (Kang et al., 2023), while another waxy sorghum exhibited an Mw value of 2.9×10^7 g/mol (Li et al., 2024), highlighting cultivar-dependent differences.

The chain length distribution (CLD) of amylopectin in sorghum starch could be accurately measured after debranching with isoamylase by using either high-performance anion exchange chromatography coupled with pulsed amperometric detector (HPAEC-PAD) or fluorophore-assisted capillary electrophoresis with a laser induced fluorescence detector (FACE-LIF) (Ai et al., 2011; Han et al., 2008; Kang et al., 2022; Sang et al., 2008). The internal structure of amylopectin consists of three types of branched chains based on Mw (Hizukuri, 1986). A-chains, the shortest, unbranched, are directly linked to B or C chains through α-1,6 glycosidic bonds, with a degree of polymerization (DP) ranging from 6 to 12. The B chains (B1 chain 13-24, B2 chain 25-36, and B3 chain DP > 37) connected to other B chains or C chains through α-1,6 glycosidic bonds. The C chain serves as the backbone of each amylopectin molecule, with only one per molecule and a reducing end (Cheetham & Tao, 1998).

The chain length distribution of amylopectin in waxy sorghum starch is shown in Table 1.1, with A-chains (DP 6-12) making up 22.6% to 35.8%, B1 chains (DP 13-24) at 41.2% to 56.2%, B2 chains (DP 25-36) at 11.2% to 14.7%, and B3 chains (DP >36) accounting for 5.4% to 11.2% (Han et al., 2008; Kang et al., 2022, 2023; Li et al., 2017; Sang et al., 2008). The average unit chain length of amylopectin was 20 to 21 glucosyl residues (Ai et al., 2011; Han et

al., 2008), and also was reported as 17 to 19 glucosyl residues (Kang et al., 2022). It should be noted that environmental factors could also influence the structure of starch, with increased growth temperatures potentially reducing branching density, depending on the variety (Li et al., 2013). Thus, adjusting these factors could further diversify sorghum starch structure.

Table 1.1 Chain length distribution of amylopectin in waxy sorghum starch.

Methods	DP 6-12	DP 13-24	DP 25-36	DP >36	Reference
HPAEC-PAD	31.1	51.4	11.6	6.0	Kang et al. (2023)
HPAEC-PAD	30.9 and 35.7	47.7 and 51.0	11.2 and 11.8	5.4 and 6.2	Kang et al. (2022)
HPAEC-PAD	35.8	41.2	11.8	11.2	Li et al. (2017)
HPAEC-PAD	43.6 (DP 6-15)	50.5 (DP 16-36)		5.8	Sang et al. (2008)
HPAEC-PAD	22.6	56.2	14.7	5.8	Han et al. (2008)

Abbreviations: DP, degree of polymerization; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection.

Functional properties of waxy sorghum starch

Hydration and swelling

Swelling of starch granules occurs after hydration, where the granules increase in size and volume as they continue to absorb water, particularly during heating. Waxy sorghum starch exhibits a two-stage swelling and solubilization pattern, which is characterized by an initial rapid phase, followed by a slight decrease, and then another rapid phase (Zahid et al., 2025). The extent of granular swelling during heating with water can be quantified as swelling power (SP), which can measure both inter- and intragranular water (Zhu, 2014). Swelling power is calculated using the following formula: Swelling Power (g/g) = W*swollen* / W*dry*, where W*swollen* is the weight of the swollen starch after centrifugation and W*dry* is the initial weight of the dry starch. The waxy starches were mostly composed of amylopectin, which could swell at lower temperatures, confirming the swelling powers of waxy starches showed higher values than those

of normal starches (Choi et al., 2004; Tester & Morrison, 1990). Table 1.2 shows the swelling power and solubilization properties of waxy sorghum starch. Studies have shown that the larger particle size and surface area of sorghum starch granules contribute to improved water solubility (Sun et al., 2014).

Table 1.2 Swelling and solubility index of sorghum starch.

Amylose (%)	SP (g/g)	SI (%)	Temperature range (°C)	Reference
7.4	45.7	24.3	85	Yan et al. (2023)
8.3	2.2-6.0	0.5-2.3	30-90	Soe Htet ea al. (2022)
5.4-6.7	3.5-7.0	95	95	Ahmed et al. (2016)
4.5	22.5	14.9	80	Akingbala and Rooney (1987)

Abbreviations: SP, swelling power, SI, solubility index

Gelatinization properties

Gelatinization is an irreversible process that involves the disruption of the molecular order within starch granules when heated in the presence of water. DSC is one of the most used methods for quantifying the physical changes of starch with water during heating and cooling (Ratnayake & Jackson, 2008). Apart from the starch itself, the results are influenced externally by the heating rate and water content (Hsieh et al., 2019; Sang et al., 2008; Zhu, 2014), which should be noted when comparing data from different studies. The gelatinization parameters by DSC usually include the onset (To), peak (Tp) and conclusion (Tc) temperatures as well as transitional enthalpy change (ΔH). The Tp was suggested to be an indicator of crystallite quality related to double helix length, and the ΔH a measure of the loss of molecular order (Sang et al., 2008). A higher degree of diversity in gelatinization properties was observed among waxy sorghum varieties (Table 1.3). The gelatinization properties of starch can be influenced by environmental conditions and genetic differences, which can lead to variations in starch

composition and structure. For example, differences in cultivation methods and environmental conditions during growth can affect the crystalline structure of the starch, thereby altering its gelatinization behavior. Waxy starches without amylose had higher gelatinization temperatures than wild types (Pedersen et al., 2007), due to the higher amount of double-helical crystallites.

The structure of amylopectin, contributing to the formation of the crystalline part in the granules, affects the gelatinization. The abundance of short unit chains (DP < 12), which may cause defective crystalline structure, could lead to lower gelatinization temperatures and transitional enthalpy change (Ai et al., 2011). In contrast, Kang et al., (2023) suggested that a decrease in longer B2- and B3-chains could potentially raise gelatinization temperature and enthalpy. The degree of crystallinity affects how much energy is required to disrupt the starch granules during gelatinization. Choi et al. (2004) reported that the enthalpy of waxy sorghum starch was higher than that of waxy millet starch, likely due to a greater amount of amylopectin in the crystalline region of starch granules, which is consistent with the data from X-ray diffractograms. Similarly, Yan et al. (2023) reported a positive correlation between relative crystallinity and ΔH, highlighting the significant influence of double-helix formation in starch on gelatinization temperature.

Table 1.3 Gelatinization properties of waxy sorghum starches determined by DSC.

Starch:water ratio (w:w)	Scanning rate (°C/min)	Gelatinization properties				Reference
		To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)	
1:3	10	73.6	77.6	82.4	14.3	Li et al. (2023)
1:3	10	73.9-74.7	78.3-78.7	82.9-83.8	17.1-18.5	Kang et al. (2022)
1:3	10	74.7-79.2	77.5-81.7	81.6-85.7	13.6-18.8	Kang et al. (2023)
1:3	10	71.8	76.5	81.3	9.5	Yan et al. (2023)
1:2	10	69.7-70.3	74.0-74.4	80.9-81.2	13.5-14.1	Ahmed et al. (2016)
1:2	10	67.7	73	82.1	14.7	Sang et al. (2008)
1:3	10	67.8	73.2	81.6	12.7	Choi et al. (2004)

To: onset temperature; Tp: peak temperature; Tc: conclusion temperature; ΔH: enthalpy of gelatinization.

Pasting properties

Pasting properties measured using the Rapid Visco Analyzer (RVA) or Brabender Visco-Amylograph (BVA) are the most reported parameters for the rheological analysis of sorghum starch. Under excess water conditions, starch is subjected to a programmed heating and cooling cycle with a constant shearing force, while viscosity development is continuously recorded (Balet et al., 2019). The total solid or water content significantly influences the rheological behavior of starch and should be considered when comparing data across different studies. Pasting profiles of waxy starches are distinct from those of their normal counterparts. Except for potato starches, waxy starches generally exhibit higher peak and breakdown viscosities, but lower setback viscosities compared to normal starches (Hsieh et al., 2019). Amylose in normal starches inhibits granule swelling and its linear molecules readily participate in reassociation during cooling stage (Chung et al., 2011; Sasaki et al., 2000).

Although significant genetic diversity in pasting properties has been observed among sorghum varieties, most studies have focused on normal sorghum, with limited research on waxy sorghum. Studies with one waxy genotype showed that the peak viscosity of waxy starch (with little amylose) was higher than nonwaxy starches (Sang et al., 2008). The pasting properties have been apparently linked to the swelling and solubilization properties (Akingbala & Rooney, 1987). The greater swelling and solubility appear responsible for the higher shear thinning behavior of sorghum starch (Subramanian et al., 1994). Choi et al. (2004) reported that starch paste of waxy sorghum composed of large-size granules gave a high viscosity compared with that of waxy millet and waxy amaranth starch with smaller granules. Pasting properties differ significantly between sorghum starch and flour, primarily due to the removal of minor constituents such as proteins and lipids during the extraction process. Irondi et al. (2022)

reported that after the removal of endogenous proteins and lipids from sorghum flour, the peak viscosity (PV) and breakdown viscosity (BV) were significantly decreased, whereas pasting temperature was increased. The reduction in PV is likely due to proteins inhibiting the swelling of starch granules during gelatinization, which significantly limits viscosity development in sorghum flour (Chanapamokkhot & Thongngam, 2007).

Retrogradation

Retrogradation refers to the process in which gelatinized starch, upon cooling below its crystalline melting point, undergoes realignment and reassociation of amylose and amylopectin polymers into more structured molecular arrangements over time (Zahid et al., 2025). In this process, amylose primarily influences the early and short-term structural changes, whereas amylopectin plays a more significant role in long-term retrogradation (Matalanis et al., 2009). Waxy sorghum primarily contains amylopectin in its endosperm starch. The long-term retrogradation properties of waxy sorghum starch is commonly measured using DSC, where gelatinized starch is stored in DSC pans for a specific duration before reheating to analyze retrogradation. Factors such as water content, storage conditions, and the structural characteristics of amylopectin significantly influence the extent of long-term behaviors. Sorghum starch with a lower proportion of short amylopectin chains with DP 6-12 and longer average chain length of amylopectin exhibited a more rapid retrogradation rate (Ai et al., 2011). Sang et al. (2008) found that after storing sorghum starch at 4 °C for seven days, normal sorghum starch exhibited a lower degree of retrogradation compared to heterowaxy and waxy sorghum starch when evaluated based on total starch weight. However, when enthalpy (ΔH) values were calculated based on the weight of the amylopectin fraction, no significant differences in retrogradation extent were detected among the three starch types. In addition, the retrogradation

properties of starches can be analyzed using time-dependent kinetic models, such as the Avrami equation, which provides the prediction of longer terms retrogradation trends based on short-term experimental data (Zhu, 2014).

Protein matrix and composition

Sorghum proteins can be broadly classified into prolamin and non-prolamin fractions. Kafirins, the major storage proteins, belong to the prolamin group and account for up to 70% of the protein in the endosperm, while non-prolamin proteins (including albumins, globulins, and glutelins) make up the remaining 30% (Bean et al., 2011). Kafirins are classified as either α, β, γ, or δ based on molecular weight and solubility. Depending on whether the sorghum endosperm is floury or vitreous, it contains approximately 66% to 84% α-kafirin, 8% to 13% β-kafirin, and 9% to 21% γ -kafirin, along with low levels of the poorly characterized δ-kafirins (Bean et al., 2019; De Mesa-Stonestreet et al., 2010). Both β- and γ-kafirins form intermolecular and intramolecular disulfide bonds and are highly crosslinked. Some previous studies show that kafirins are located primarily in spherical protein bodies, which are embedded in a glutelin protein matrix, and are surrounded by starch granules (Choi et al., 2008; De Mesa-Stonestreet et al., 2010). The protein bodies range from 0.4 to 2 μm in diameter, with an outer layer primarily composed of crosslinked β- and γ-kafirins, while the interior is mainly composed of α-kafirin. Sorghum glutelin functions as a structural component within the matrix of the peripheral and inner endosperm and may also provide enzymes for starch and protein hydrolysis (Bean et al., 2011).

The microstructure of kafirins in relation to the glutelin protein matrix and starch granule have been studied using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). The CLSM images of uncooked native sorghum and cooked sorghum samples show that starch granules are encapsulated within a protein matrix in the uncooked state

(Choi et al., 2008). After cooking, intra- or intermolecular exchange of disulfide bonds may increase the tightness in the sorghum flour. In addition, Hamaker & Bugusu (2003) observed the protein bodies as small circular bodies embedded in a larger protein matrix using CLSM with 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde (CBQCA) as the protein dye. After cooking, sorghum proteins appear to form extended web-like or sheet-like structures with starch embedded within. This structural formation during cooking could impact functional and nutritional properties of sorghum-based products. Further research is needed to understand and improve the role of sorghum proteins in food functionality, particularly for food production in western countries.

Application of waxy sorghum

The use of sorghum grain varies widely by region. In many parts of the world, it is a staple food for millions of people and consumed in various traditional food and beverage products. However, in the United States, sorghum is mainly used for animal feed and is increasingly utilized in ethanol production for the biofuels market (Bean et al., 2019). Previous reviews have summarized the food and nonfood uses of sorghum (McGinnis & Webster, 2024; Taylor et al., 2006). The potential starch-related applications of waxy sorghum are diverse, including foods and alcoholic beverages, thickening agent, packaging materials, substrate for bacteria, and biofuel (ethanol) production (Kang et al., 2022; Kwon et al., 2005; McGinnis & Webster, 2024; Zhu, 2014). Waxy sorghum is recognized as an important feedstock for ethanol production due to its high amylopectin content, leading to higher ethanol conversion efficiency compared to non-waxy varieties. Its advantages in ethanol production include higher starch and protein digestibility, easier starch gelatinization, lower viscosity during liquefaction, shorter fermentation times, and increased free amino nitrogen levels that support fermentation (Lu et al.,

2013). Sattler et al. (2009) also noted that waxy sorghum has higher ethanol conversion rates than non-waxy varieties due to the absence of amylose, which prevents the formation of amylose-lipid complexes that resist enzymatic breakdown during fermentation. The increased ethanol yield from waxy sorghum also suggests reduced processing time and energy requirements, potentially lowering costs in industrial biofuel production. Waxy sorghum is traditionally used in the production of liquor, especially in China, and it serves as a key ingredient in the fermentation process of famous Chinese liquors such as Maotai. The ease of gelatinization and low viscosity of waxy sorghum starch during processing is crucial for efficient fermentation (Lu et al., 2013). Waxy sorghum starch exhibits significantly higher starch and protein digestibility than non-waxy sorghum, making it suitable for food products that require enhanced digestibility, such as processed foods and formulations for infants or the elderly. Elhassan et al. (2015) reported that waxy sorghum varieties with high protein digestibility demonstrated greater solubility and paste viscosity, closely similar to the properties of wheat flour used in commercial bread production.

Objectives

The goal of this research is to identify unique properties and define differentiated value for waxy sorghum flour and starch as food ingredients. Waxy sorghum starch has potential, but we do not know how starches in different waxy sorghum grains are compared with waxy maize starch. Additionally, waxy sorghum flour could be used as a natural ingredient in foods where chemically modified starches are banned. The specific objectives were to

(1) develop an efficient enzymatic treatment for isolating starch from decorticated or whole waxy sorghum grains with high yield and purity and evaluate its recovery, purity, and functional properties in comparison to the conventional wet milling.

(2) identify waxy sorghum starches and flours with higher thickening power and greater freezing-thawing stabilities in comparison with waxy maize starch, examine how the pasting and retrogradation properties of starch are affected by the structural properties (chain-length distribution and molecular distribution), and investigate the correlations between starch structural features, functional properties of starches and flours, and their compositional characteristics.

(3) develop processes to improve functional properties of waxy sorghum flours. One specific aim was to examine how protein matrix in waxy sorghum flours affect the stability and pasting of cooked waxy sorghum flours under shear.

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Chapter 2 - Comparison of Three Methods to Isolate Starch from Waxy Sorghum Grains

Abstract

Waxy sorghum contains essentially only amylopectin in its endosperm starch and has properties that could be potentially used in value-added food ingredients as an effective thickener. The objectives of this study were to develop an efficient process to isolate waxy sorghum starch and compare the properties of the waxy sorghum starches isolated by enzymatic processes and traditional wet milling. Alkaline protease was utilized to isolate starch from decorticated and whole waxy sorghum grains, providing a comparative analysis against the conventional wet milling. To evaluate the efficiency of the isolation process, key parameters such as starch recovery, yield, residual protein, ash, damaged starch, color, as well as pasting and thermal properties were determined. Starch recovery showed no statistically significant differences among the three methods, and all isolated starches exhibited desired purity regardless of the method employed. However, enzymatic treatment methods not only produced starches with lower residual protein (0.18-0.22%) but also resulted in significantly greater peak (1992-2023 cP), breakdown (1123-1172 cP), and setback viscosities (275-355 cP) compared with the waxy sorghum starch isolated by the wet milling. The starches isolated by wet milling and enzymatic treatment of decorticated sorghum exhibited similar onset and peak gelatinization temperatures, while both methods produced starches with better color quality (higher L*, lower b*) compared to starches isolated from whole sorghum using the enzymatic treatment. As a result, enzymatic treatment of decorticated sorghum could be used as an effective method for starch isolation at the laboratory scale, with promising potential for application in industrial-scale

processes.

Introduction

Sorghum (*Sorghum bicolor* L. Moench) ranks as the 5th most important cereal crop globally, following corn, rice, wheat, and barley (Bean et al., 2019). Due to its low production costs, drought resistance, and tolerance to high temperatures, sorghum plays a critical role in food security in some semiarid areas of Asia, Africa, and South America (Bean et al., 2019; Beta et al., 2004; Dicko et al., 2006; Shaikh et al., 2024). Starch, the primary storage carbohydrate in sorghum grains, constitutes approximately 70% of the dry grain weight (Liu et al., 2012; Sang et al., 2008) and is widely used in both food and non-food applications (Deshpande & Panya, 1987; Ai et al., 2011; Awika & Rooney, 2004; Glennie, 1988; Shaikh et al., 2024; Yan et al., 2011). Many important physicochemical and nutritional properties of starch are influenced by the ratio of amylose and amylopectin, the two major polymers in the starch granule (Bean et al., 2019; Zhu, 2014). Waxy sorghum contains essentially only amylopectin in its endosperm starch and has unique properties that could be used as value-added food ingredients and increase its value compared with normal sorghum (Hsieh et al., 2019a). In general, amylose prompts the gelling and film-forming properties of starch and creates highly associated starch. Amylopectin, on the other hand, increases the thickening power of starch and promotes the clarity of starch pastes and gels, which are crucial for controlling the consistencies of soups, gravies, sauces, and fillings (Hsieh et al., 2019a; Sang et al., 2008). Currently, waxy maize starch is the preferred base starch, which is further chemically processed and modified to produce food thickeners (Rapaille & Vanhemelrijck, 1992; Reddy & Seib, 2000). However, clean label is an emerging global trend in packaged foods, and waxy sorghum starch has the potential to be directly used as an effective

thickener in food applications. This could benefit the economic development of countries where sorghum is the primary cereal crop (Easterly & Levine, 2003).

To enhance the use of sorghum starch in food, feeds, and industrial applications, it is necessary to develop a highly effective starch isolation process while minimizing complexity and cost. The wet milling process is commonly used for sorghum starch production on an industrial scale (Eckhoff & Watson, 2009), involving grain cleaning, sulfur dioxide steeping, grinding, separating components with hydrocyclones, and purifying and drying the starch. A variety of methods have been tested to improve sorghum starch isolation at the laboratory scale, including the application of alkali solutions, sulfite treatments, lactic acid, and hydrolytic enzymes, individually or in combination, to separate starch from proteins, fibers, and lipids (Ahmed et al., 2016; Eckhoff & Watson, 2009; Higiro et al., 2003; Micaela & Drago, 2020; Pérez Sira & Lares Amaiz, 2004; Beta et al., 2000; Wang et al., 2000; Xie & Seib, 2000). In a conventional wet milling process, the sorghum grain is first steeped in water containing sulfur dioxide (SO_2) as a reducing agent and lactic acid for 24 to 48 hours (Buffo et al., 1998; Cruz et al., 2015; Higiro et al., 2003; Xie & Seib, 2000). The addition of lactic acid significantly increased starch yield compared to sulfur dioxide (SO_2) alone (Pérez et al., 2001) while also controlling microbial growth by reducing the pH of the steeping solution (Wang et al., 2000). However, effective separation of sorghum starch is challenging due to the presence of polyphenols like tannins in some varieties that give off color to the resultant starch (Zhu, 2014). Some studies reported that alkaline treatments (NaOH) effectively improved starch brightness and whiteness by reducing phenolic compounds (Beta et al., 2001; Yang & Seib, 1996), while decortication prior to wet milling could also reduce pigmentation by removing the outer layers of sorghum grains (Higiro et al., 2003). Pérez Sira & Lares Amaiz (2004) explored the use of alkaline reagents, including a

mixture of sodium hypochlorite and potassium hydroxide, to bleach starch and effectively remove phenolic compounds responsible for discoloration. This method may raise environmental and safety concerns due to the use of chemicals like sodium hypochlorite and potential residues from the bleaching process. High-intensity sonication offered a rapid approach with minimal chemical use, producing high-purity starch; however, it required specialized equipment and may damage starch granules during the process (Benmoussa & Hamaker, 2011; Park et al., 2006; Qi et al., 2018).

Protease enzymes have been used in the isolation of cereal starch to achieve higher-purity starch with lower residual protein content (Chew-Guevara et al., 2016; Dorglamud et al., 2013; Lim et al., 1992; Lumdubwong & Seib, 2000; Sittipod & Shi, 2016; Tester et al., 2007, 2008; Zhu et al., 2010). Lumdubwong & Seib (2000) investigated the use of a food-grade alkaline protease to isolate rice starch from wet-milled rice flour, achieving a starch recovery of 95% and a residual protein content of 0.5% after treatment at pH 10.0 for 18 hours. Wang & Wang (2001) evaluated the effectiveness of acid, neutral, and alkaline protease treatments for rice starch isolation and compared them with the conventional alkaline steeping method. Their findings revealed that alkaline protease treatment achieved the highest starch yield (77.6%) and desired protein residue (≤0.2%), along with higher peak and final viscosities compared to starch isolated using acidic or neutral protease treatments. In addition, Radosavljevic et al. (1998) reported that using alkaline protease in combination with low-concentration NaOH steeping significantly reduced protein residue levels (≤0.2%) and improved the recovery (~80%) of amaranth starch. Although the starch and protein matrix in sorghum interact strongly, protease treatment has been shown to effectively hydrolyze protein and release starch granules (De Mesa-Stonestreet et al., 2010; Mezo-Villanueva & Serna-Saldívar, 2004). Chew-Guevara et al. (2016) reported that the

combination of decortication and neutral protease (Neutrase®) treatment significantly increased the starch yield from 71.9% to 91.8% and decreased the residual protein from 0.51% to 0.12%, compared to isolation process without these treatments. Additionally, they found that the starch isolated from red sorghum (non-waxy) by decortication and protease treatment exhibited significantly lower pasting temperature, higher peak, and lower final viscosities compared to starch isolated without these treatments.

Variations in isolation methods and conditions can significantly influence starch composition and functional properties (Beta et al., 2001; Qi et al., 2018; Wang et al., 2000), possibly providing opportunities to regulate the functional properties of starch. To scale up production to a pilot plant level, a starch isolation process must feature a high yield, fine color, minimal input requirements, and cost-effective operation (Palavecino et al., 2019). While extensive research has been conducted on sorghum starch isolation, studies that compare the purity and functional properties of starch isolated from waxy sorghum grains using traditional wet milling and enzymatic treatment methods remain limited. Moreover, the effectiveness and impact of alkaline protease treatment on starch isolation from both decorticated and whole waxy sorghum have not been fully investigated. The objectives of this study were to develop enzymatic methods for isolating starch from whole and decorticated waxy sorghum with high yields utilizing alkaline protease, to evaluate its recovery, purity, and functional properties in comparison to the conventional wet milling, to investigate the underlying factors contributing to variations in the quality and functional properties of isolated starches, thereby evaluating its potential for large-scale production.

Materials and methods

Materials

Three waxy grain sorghum varieties, grown in Assaria, Kansas, were obtained from the Kansas State Center for Sorghum Improvement (Table 2.1). All sorghum samples were cleaned by hand to remove foreign materials and then stored at room temperature. Coarse grinding of steeped sorghum grains was performed using a Waring blender (1L, model 5011, Dynamic Corp. of America, New Hartford City, CT) for all isolation methods. Fine grinding was performed using a plate mill (Quaker City, model No. 4E, Straub Co., Warminster, PA) specifically designed for wet milling. The sieves used were No. 16 and 200 wire mesh from Fisher Scientific (Fair Lawn, NJ, USA). The Danisco FoodPro alkaline protease (specific activity 580,000 to 650,000 DU/g) was supplied by DuPont Nutrition and Health (Rochester, New York, USA). The starch table (244 cm long, 5.08 cm wide, and 2.54 cm high with rounded internal corners), an aluminum I-beam, was designed and manufactured by the Department of Biological and Agricultural Engineering at Kansas State University (Xie & Seib, 2000). All other chemicals were analytical grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Methods

Preparation of decorticated sorghum grains

Waxy sorghum grains were decorticated using a tangential abrasive dehulling device (Venables Machine Works, Saskatoon, SK, Canada) equipped with an 80-grit abrasive disk, removing approximately 20% of the bran and germ by weight as described by Park et al. (2006). Decorticated sorghum grains were then used to isolate starch through enzymatic treatment.

Isolation of sorghum starch

Three laboratory methods were employed and evaluated for isolating starch from grain sorghum. The yield (Y%) and recovery (R%) of starch were calculated using Equations (1) and (2) below, respectively, based on the dry weight of starch and sorghum grains.

Y% = (Isolated starch, g / sorghum grain weight, g) * 100 *(Equation 1)*

R% = (Isolated starch, g / total starch in sorghum grains, g) * 100 *(Equation 2)*

Wet milling

Sorghum starch was isolated following the procedure described by Xie & Seib (2000) with modifications (Figure. 2.1). The whole sorghum grains were steeped in a water bath at 50°C for 48 hours (Micaela & Drago, 2020; Qi et al., 2018; Wang et al., 2000). After steeping, the steep liquor was decanted, and the grains were coarsely ground using a Waring blender. The resulting slurry was passed through a No. 16 wire sieve (1mm), and fine grinding was done on a plate mill. The ground mixture was then filtered through a No. 200 wire sieve (75 μm) to remove fine fiber. Starch and protein separation was achieved by using a starch table with a slope of 1.0%. The starch was left to dry overnight on the table and was collected using a plastic scraper.

Enzymatic treatment of decorticated or whole sorghum grains

The enzymatic treatment methods for starch isolation from decorticated or whole sorghum grains follow the same procedure, differing only in the starting materials (Figure. 2.2). Decorticated or whole sorghum grains (100 g, dry basis) were added into an Erlenmeyer flask with 200 mL of distilled water containing 0.32% (w/v) sodium bisulfite and 0.55% (v/v) lactic acid. The flask was stoppered and incubated in a water bath at 50°C for 48 hours, after which the steep liquor was decanted. The steeped sorghum kernels were rinsed with 350 mL of water.

Subsequently, the steeped sorghum with water (≈150 mL) was transferred to a Waring blender and ground at low speed for 10 min, with the temperature carefully maintained below 40 °C to prevent gelatinization. The slurry was poured through a No. 200 wire sieve (75 μm) and the pH was adjusted to 8.0 using 2.0% (w/v) NaOH solution (avoid starch gelatinization). To hydrolyze sorghum protein, 0.5%(w/v) FoodPro alkaline protease, based on the weight of sorghum grains, was added to the slurry. During protein hydrolysis, the pH was continuously monitored and maintained at a constant level of 8.0 while the reaction proceeded at 45°C for 4 hours. Subsequently, the pH was decreased to 4.0 by adding 1M HCl to deactivate the protease for 30 min and then adjusted to 6.0 using 2% (w/v) NaOH. The slurry was centrifuged three times at 3000 ×g for 20 min. If a dark layer appeared on top of the sediment after centrifugation, it was carefully removed. The sediment (starch cake) was then scraped off using a spatula and dried in a convection oven at 40 °C for 24 h.

Moisture, ash, protein, total starch and damaged starch content

The moisture content of isolated sorghum starch was measured using AACC Approved Method 44-15A, and the ash content was determined according to AACC Approved Methods 08-01. Protein content was measured using a nitrogen determinator (LECO FP-828, St. Joseph, ML) following the AACC 46-30.01 method, with a conversion factor of 6.25 for nitrogen to protein. Total starch and damaged starch contents were determined using commercial kits from Megazyme (Wicklow, Ireland), following AACC Approved Methods 76-12 and 76-31, respectively.

Color analysis

The color of starch samples was measured using a colorimeter (Konica Minolta, Model: BC-10, Japan). The results were expressed as L* (lightness) with a scale ranging from 0 (black)

to 100 (white), a* value (redness), ranging from +60 (red) to -60 (green), and b* value (yellowness), ranging from +60 (yellow) to -60 (blue). Each sample was analyzed in triplicate to ensure accuracy.

Pasting properties

The pasting properties of isolated starches were determined using a Rapid Visco Analyzer (RVA-4500, Perten Instruments, Sweden). A 6% starch suspension was prepared by mixing 1.68g of starch (dry basis) with 26.32g of distilled water (corrected by sample moisture). The measurements were conducted following the STD2 temperature profile (Approved Method 76-21.02, Cereals& Grains Association, 2009). The sample was initially held at 50 °C for 1 min, heated to 95 °C in 7.5 min, maintained at 95 °C for 5 min, cooled to 50 °C in 7.5 min, and then held at 50 °C for an additional 2 min. The rotating speed of the paddle was set at 160 rpm. The values of pasting temperature, peak, breakdown, setback, and final viscosities were recorded.

Thermal properties

The gelatinization properties of the isolated starches were determined by differential scanning calorimetry (DSC) at the ratio of starch to water (1.0/1.5, dry basis), following the procedure described by Shi & Seib (1992). Starch samples (10 mg) were precisely weighed into stainless-steel pans, mixed with 15mg of distilled water (corrected by sample moisture), and sealed, while an empty pan was used as a reference. The gelatinization properties were conducted using a Q200 DSC (TA Instrument, New Castle, DE, USA) by heating the samples at 10 °C / min from 10 °C to 120 °C. The onset, peak, and conclusion temperatures (To, Tp, and Tc, respectively), as well as endothermic enthalpy (ΔH), were calculated from the DSC thermogram by Universal Analysis software (TA Instrument, New Castle, DE, USA).

Molecular size distribution of whole sorghum starch

The molecular size distribution of isolated starches was determined by gel permeation chromatography (GPC) (PL-GPC 220, Polymer Laboratories Varian, Inc. Amherst, MA, USA) coupled to a refractive index (RI) detector, based on the method of Shi et al. (2018). Starch samples (8 mg, dry basis) were dissolved in 8 ml of dimethyl sulfoxide (DMSO) containing 0.5% (w/v) lithium bromide (LiBr) and heated in a boiling water bath with stirring for 24h to ensure complete dissolution. Once fully dissolved, a 1.0-1.5 ml aliquot of the sample solution was filtered through a 2 μm filter into GPC vials and loaded into the autosampler. The eluent used was DMSO with 0.5% LiBr, delivered at a flow rate of 0.8 ml/min, with the temperature maintained at 80 °C throughout the analysis. Calibration of the GPC system was performed using Pullulan standards (molecular weights ranging from 342 to 6.36×10^5 Da) supplied by Showa Denko K.K. (Tokyo, Japan), following the procedure described by Tizzotti et al. (2013). The GPC data were processed using Cirrus™ GPC software (version 3.0, Agilent Technologies, Santa Clara, CA, USA).

Statistical analysis

Color measurement was performed in triplicate. All other experiments were done in duplicate. Collected data were analyzed using IBM SPSS Statistics Version 26 (IBM Corporation, New York, USA). Analysis of variance (ANOVA) was applied to determine whether data differed significantly from each other ($p < 0.05$). The results were expressed as mean and standard deviations (SD).

Results and Discussion

Decortication of waxy sorghum grains

To examine the effects of sorghum pericarp on isolation of starch, decorticated sorghum grains were prepared prior to the protease treatment. Waxy sorghum samples were decorticated to approximately 20% (by weight), increasing starch content from 72-73% in whole sorghum grains to 83-84% in the decorticated grains (Table 2.1). The decortication of waxy sorghum grains resulted in a 7-8% loss of starch (dry basis), which is attributed to the mechanical removal of outer grain layers that may also include portions of the endosperm containing starch granules. Additionally, some starch loss may be due to the removal of starch-containing bran during decortication. Sorghum is classified by color into white, yellow/red, and brown or high-tannin types, where pigments are primarily concentrated in the pericarp, testa, and aleurone layers (Chiremba et al., 2012; Pérez Sira & Lares Amaiz, 2004). These pigments could contaminate starch during starch isolation processes, reducing their value in food applications (Beta, Corke, Rooney, et al., 2001). Decortication could improve the penetration of sulfur dioxide into the decorticated kernels, promoting the enzymatic hydrolysis of endosperm protein structures by proteases (Aboubacar et al., 2006; Duodu et al., 2003). We should note that the traditional wet milling method used in this study, as described by Xie & Seib (2000), did not involve decortication and instead relied on screening and a starch table to separate starch from protein and other non-starch components. In this study, we examined how the decortication prior to protease treatment affects starch recovery, color, and the functional properties of the resulting starches, in comparison to starches obtained by using the wet milling method.

Starch isolation and the relationship between starch recovery and grinding time

The relationship between starch recovery and grinding time is shown in Figure 2.3. The recovery and purity of sorghum starch are influenced by the physical properties of the grain, steeping and grinding conditions, and the extent of decortication (Anglani, 1998; Eckhoff & Watson, 2009; Xie & Seib, 2000; Yang & Seib, 1996). Optimizing these factors can improve yield and starch quality, making sorghum a competitive source for industrial starch production. Starch recovery significantly increased with longer grinding times, likely due to changes in the particle size distribution of the slurry (Fig. 2B&C). Longer grinding time resulted in a decreased proportion of large particles and an increased proportion of smaller particles, facilitating the release of more starch granules embedded within or associated with non-starch components. However, extending the grinding duration from 10 to 15 minutes resulted in no further improvement in starch recovery, likely because most starch granules had already been released by 10 minutes, as indicated by the mostly unchanged proportion of particle size around 20 μm, comparable to the size of sorghum starch granules. Avoiding unnecessary grinding time is essential to prevent alterations in the physicochemical properties of starch and to minimize energy consumption (Liu et al., 2018). As a result, a grinding time of 10 minutes was selected as the optimal duration for starch isolation from waxy sorghum across all three methods.

The yield, recovery, and purity of isolated starch using three different methods are summarized in Table 2.2. The enzymatic treatment of decorticated sorghum had a significantly higher starch yield (78.8-79.1%) compared to the other two methods (63.6-64.1%) due to the removal of the outer layers of sorghum grains which increased the total starch available for extraction. The starch recovery from the three methods showed no statistical differences, with values ranging from 86.6-88.3%. In this study, the starch recovery using traditional wet milling

was higher compared to the results (~70%) reported by Chew-Guevara et al. (2016) and Micaela & Drago (2020) for wet milling. However, Chew-Guevara et al. (2016) reported that starch recovery increased to 91.8% with the use of decortication and neutral protease treatment, which is approximately 4% higher than our results. The difference may be due to our study accounting for starch loss during decortication, whereas the previous study did not specifically mention whether starch loss from decortication was accounted for in starch recovery calculations.

All starches achieved the desired purity, regardless of the isolation method, with ash and protein contents below 0.180% and 0.41%, respectively (Table 2.2). The damaged starch content in all samples remained below 1.4%, aligning with the results reported by Micaela & Drago (2020) and lower than the results reported by Park et al. (2006). Starch isolated through enzymatic treatment of decorticated sorghum exhibited slightly increased damaged starch content, likely due to the mechanical forces applied during decortication. The gradual abrasion of the outer layers exposed starch granules to high shear forces and heat generation, potentially resulting in the damage and alteration of starch granules (Buitimea-Cantúa et al., 2013). Starch isolated using enzymatic treatment exhibited significantly lower protein content (0.18-0.22%) compared to the wet milling process (0.36-0.41%), which agreed with previous studies that the isolated starch contained approximately 0.2% residual protein following protease treatment (Chew-Guevara et al., 2016; Radosavljevic et al., 1998; Wang & Wang, 2001). These results indicate that the protein matrices in waxy sorghum were effectively disrupted by alkaline protease, releasing a greater amount of starch granules during protein hydrolysis (Barros et al., 2012). However, starch isolated through wet milling may retain tightly bound kafirin, which could influence its swelling and pasting properties.

Color properties

Color is a crucial factor in evaluating starch quality and consumer acceptance, with higher *L* values and lower *a* and *b* values reflecting greater purity (Wang et al., 2000; Zambelli et al., 2024). The color parameters of starch (in its dry powder form) obtained through three isolation methods are presented in Table 2.2. The wide variation in seed colors among sorghum cultivars can lead to pigment contamination of starch during starch isolation, reducing its suitability for food applications (Boudries et al., 2009; Pérez Sira & Lares Amaiz, 2004; Qi et al., 2018). In this study, when enzymatic treatment was applied to isolate starch from whole sorghum grains, pigments from the sorghum bran contaminated the starch during steeping and protein hydrolysis, forming a thick and dark color within the whole sediment after centrifugation, making it challenging to clean and separate (Beta et al., 2001; Chew-Guevara et al., 2016). As a result, the isolated starch exhibited significantly lower *L* values (95.8-96.4) and higher *b* values (4.2-4.6) compared to the other two methods, resulting in a less desirable appearance. However, the starch isolated through enzymatic treatment of decorticated sorghum exhibited a higher *L* value (99.1-98.2) and a similar *b* value (3.1-3.2) to that of starch obtained by wet milling (*L*, 97.5-98.6; *b*, 3.1-3.3). Therefore, removing the outer layer of sorghum grains through decortication improves the appearance and quality of starch, making it more suitable for commercial purposes.

Pasting properties

The pasting profiles and corresponding attributes of waxy sorghum starches are shown in Figure 2.4 and Table 2.3, respectively. Waxy starches are known to have higher peak viscosities than amylose-containing starches, followed by a more pronounced breakdown and a lower degree of setback after pasting (Garimella Purna et al., 2015; Hsieh et al., 2019a, 2019b; Wang et

al., 2018). Amylose molecules in normal sorghum starches could inhibit the swelling of starch granules by forming complexes with lipids, resulting in a reduced peak viscosity and higher pasting temperature (Garimella Purna et al., 2015; Sang et al., 2008). Some studies have shown that variations in isolation methods and conditions can significantly impact the functional properties of starch (Belhadi et al., 2013; Benmoussa & Hamaker, 2011; Beta, Corke, Taylor, et al., 2001; Qi et al., 2018; Wang et al., 2000). In our study, the pasting temperatures of starches isolated using the three methods showed no statistical differences. However, starches isolated through enzymatic treatments exhibited significantly greater peak (1992-2006 cP), breakdown (1123-1172 cP), and setback (275-355 cP) viscosities compared to the conventional wet milling process (1910-1920 cP, 970-986 cP, 195-218 cP, respectively). This implied that protease treatment effectively degraded the residual proteins tightly associated with waxy sorghum starch granules, which were challenging to completely remove through wet milling, resulting in more residual protein that limited the swelling capacity of the starch granules and reduced peak viscosity (Barros et al., 2012). Chew-Guevara et al. (2016) reported that starch isolated from red sorghum (non-waxy) using neutral protease treatment exhibited significantly higher peak and lower final viscosities compared to starch isolated without these treatments. In addition, Lim et al. (1999) found that residual protein content played a critical role in determining the pasting properties of rice starch, with lower residual protein leading to increased peak and breakdown viscosities.

As previously mentioned, extending the grinding time from 10 to 15 minutes resulted in minimal or no further improvement in starch yield and recovery. The pasting properties of sorghum starch (F-019) isolated using three methods with shorter (10 min) and longer (15 min) grinding times were evaluated (Figure. 2.5 and Table 2.5). No significant difference was

observed in peak, breakdown, and final viscosities of starch obtained with different grinding times ($p < 0.05$), regardless of the method used. In addition, the pasting properties of starch isolated by wet milling under two steeping temperatures (50°C and 52°C) were investigated (Figure 2.4 and Table 2.3). Qi et al. (2018) reported that 50°C was the optimal steeping temperature for sorghum grains and this temperature also utilized in other studies for sorghum starch isolation (Mezo-Villanueva & Serna-Saldívar, 2004; Wang et al., 2000). We did not find significant differences in peak and setback viscosities of the starches isolated using those two steeping temperatures, confirming that 50-52°C were optimal steeping temperatures for isolation of starch from the sorghum grains. Higher steeping temperatures were not used because higher temperatures may risk partially gelatinizing starch in sorghum grains, which would negatively affect the recovery of starch.

Thermal properties

The gelatinization temperatures (To, Tp, and Tc) and enthalpies (ΔH) of waxy sorghum starches are presented in Table 2.5. Regardless of the isolation method, F-032 and F-037 exhibited higher onset (To) and peak (Tp) gelatinization temperatures, as well as greater enthalpies (ΔH) in comparison with F-019, indicating that F-032 and F-037 formed stronger crystalline structures and greater molecular order, which are related to double helix length (Campanha & Franco, 2011; Cooke & Gidley, 1992). Some previous studies have reported that the gelatinization characteristics of sorghum starch from the same variety may vary depending on the extraction methods (Chew-Guevara et al., 2016; Micaela & Drago, 2020). In this study, starch isolated by enzymatic treatment of whole sorghum exhibited a slightly higher onset temperature (73.1-74.5°C) and a similar peak temperature (77.3-78.9°C) compared to the other two methods. This increase in onset temperature was likely due to the lower damaged starch

content (Table 2.2), as intact starch granules require more thermal energy to disrupt their crystalline structure (Ratnayake & Jackson, 2008). All starches isolated from the three varieties of waxy sorghum using enzymatic treatment methods had slightly higher gelatinization enthalpy values (20.7-21.8 J/g) compared to the starches isolated by the wet milling process (20.2-21.1 J/g) (Table 2.5).

Molecular distribution of waxy sorghum starches

The molecular size distribution (expressed as retention time) of whole waxy sorghum starches is shown in Figure 2.5. The gel-permeation chromatography (GPC) technique separates molecules based on their size in the eluent (Hsieh et al., 2019; Shi et al., 2018), allowing for the determination of the distribution of whole starch molecules without breaking them down, preserving the natural branched structure of amylopectin and linear structure of amylose. Non-waxy whole cereal starches typically exhibit a bimodal distribution with two distinct peaks in the GPC chromatogram, representing amylopectin (larger size, highly branched molecules) and amylose (smaller size, linear molecules with few branches) (Lin et al., 2017; Tester & Karkalas, 1996). In this study, waxy whole sorghum starches exhibited a single peak corresponding to amylopectin, with similar retention time, peak area, and peak shape regardless of the isolation method (Figure 2.5), suggesting that molecular structure of amylopectin was not significantly different in the starches isolated by different methods.

Comparison of three starch isolation methods for waxy sorghum grains

Three starch isolation methods for waxy sorghum grains are compared and summarized in Table 4. Starch recovery showed no statistical differences among the three methods, ranging from 86.6% to 88.3%. All starches isolated had ash and protein contents below 0.18% and 0.41%, respectively. The starches isolated by wet milling and enzymatic treatment of

decorticated sorghum exhibited similar onset and peak gelatinization temperatures, while both methods produced starches with better color quality (higher L and lower b values) compared to the starches isolated by the enzymatic treatment of whole sorghum. However, the enzymatic treatment of decorticated sorghum not only produced starch with significantly lower protein residues (0.18-0.22%) compared to the starches by the wet milling (0.36-0.41%) but also resulted in significantly greater peak, breakdown, and setback viscosities in comparison with the starches by the wet milling.

Conclusion

An efficient process was developed to isolate waxy sorghum starch by using alkaline protease, and its physicochemical properties were compared with the traditional wet milling method. Overall, enzymatic treatment of decorticated sorghum could be used as an effective and efficient method for starch isolation, producing starch with higher purity, better color quality, and enhanced pasting viscosities, making it a promising material for application as a food thickener

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Table 2.1 Hardness, diameter, and weight of three sorghum grains; and total starch content of whole sorghum and decorticated sorghum grains.

Sample	Product Name	Grain hardness index	Grain diameter (mm)	Grain weight (mg)	Total starch%		Starch loss (%)
					Sorghum grains	Decorticated sorghum grains	
F-032	X20211	75.48	2.49	24.58	72.9±0.3	83.4±0.9	8.5
F-037	X20213	65.80	3.01	35.26	72.7±0.3	84.4±0.6	7.1
F-019	X20207	70.32	2.76	30.09	72.4±0.3	83.2±0.2	7.9

*Starch loss (%) refers to the starch lost from sorghum grains during the decortication process.

Table 2.2 Yield and recovery, composition, damaged starch content, and color of sorghum starches isolated by enzymatic treatment and wet milling methods.

Sample	Yield (%)	Recovery (%)	Ash (%)	Protein (%)	Damaged Starch (%)	Color		
						L*	a*	b*
	Enzymatic treatment of decorticated sorghum							
F-032	78.9±0.5^{a}	86.6±0.5^{a}	0.115±0.005^{d}	0.19±0.02^{b}	1.40±0.02^{a}	99.1±0.1^{a}	-0.2±0.1^{e}	3.2±0.0^{c}
F-037	79.1±0.4^{a}	87.0±0.4^{a}	0.125±0.018^{cd}	0.18±0.01^{b}	1.39±0.01^{a}	98.3±0.3^{b}	-0.5±0.0^{f}	3.2±0.2^{c}
F-019	78.8±0.7^{a}	87.1±0.8^{a}	0.128±0.008^{bcd}	0.18±0.01^{b}	1.37±0.01^{a}	98.2±0.2^{b}	0.0±0.1^{d}	2.9±0.2^{c}
	Enzymatic treatment of whole sorghum							
F-032	63.6±0.4^{b}	87.2±0.6^{a}	0.144±0.003^{abcd}	0.21±0.05^{b}	1.16±0.01^{c}	95.8±0.2^{e}	0.7±0.1^{a}	4.6±0.2^{a}
F-037	63.8±0.4^{b}	87.8±0.6^{a}	0.153±0.006^{abcd}	0.22±0.02^{b}	1.10±0.01^{d}	96.0±0.3^{de}	0.3±0.0^{b}	4.2±0.1^{b}
F-019	64.0±0.6^{b}	88.3±0.9^{a}	0.152±0.011^{abcd}	0.19±0.06^{b}	1.14±0.02^{cd}	96.4±0.2^{d}	0.7±0.0^{a}	4.6±0.1^{a}
	Wet milling							
F-032	63.8±0.4^{b}	87.6±0.5^{a}	0.179±0.010^{a}	0.36±0.02^{a}	1.26±0.02^{b}	98.6±0.1^{b}	0.3±0.1^{b}	3.1±0.1^{c}
F-037	64.1±0.5^{b}	88.1±0.7^{a}	0.174±0.016^{ab}	0.37±0.04^{a}	1.27±0.01^{b}	97.5±0.1^{c}	0.3±0.0^{b}	3.1±0.1^{c}
F-019	63.6±0.4^{b}	87.8±0.6^{a}	0.169±0.021^{abc}	0.32±0.02^{a}	1.25±0.01^{b}	97.6±0.1^{c}	0.2±0.1^{c}	3.3±0.1^{c}

* Starch loss during the decortication process is also considered to calculate the recovery; L*, a*, and b* represent lightness, redness, and yellowness, respectively.

*Mean values of two measurements; values followed by the same letters in the same column are not significantly different ($p < 0.05$).

Table 2.3 Pasting properties of sorghum starches isolated by enzymatic treatment and wet milling methods.

Sample	Pasting temperature	Peak viscosity	Breakdown viscosity	Setback viscosity	Final viscosity
	(°C)	(cP)	(cP)	(cP)	(cP)
	Enzymatic treatment of decorticated sorghum				
F-032	77.4±0.3^{a}	2006±8^{a}	1143±10^{b}	355±7^{a}	1218±6^{a}
F-037	77.2±0.1^{a}	2003±3^{a}	1139±2^{b}	343±5^{ab}	1207±6^{ab}
F-019	77.3±0.3^{a}	2006±4^{a}	1129±4^{b}	328±6^{bc}	1205±6^{ab}
	Enzymatic treatment of whole sorghum				
F-032	77.6±0.1^{a}	2023±1^{a}	1172±3^{a}	275±4^{e}	1125±7^{h}
F-037	77.8±0.1^{a}	1987±16^{a}	1128±6^{b}	301±2^{d}	1160±11^{defg}
F-019	77.7±0.1^{a}	1992±6^{a}	1123±2^{b}	314±4^{cd}	1178±8^{bcde}
	Wet milling under 50°C				
F-032	77.7±0.2^{a}	1913±1^{bc}	986±3^{c}	212±9^{fg}	1139±13^{fgh}
F-037	77.4±0.2^{a}	1920±1^{b}	984±6^{c}	218±2^{f}	1153±8^{efgh}
F-019	77.5±0.0^{a}	1910±11^{bc}	970±12^{c}	195±1^{g}	1135±0^{gh}
	Wet milling under 52°C				
F-032	77.8±0.4^{a}	1922±6^{b}	940±7^{d}	215±0^{f}	1197±1^{abc}
F-037	77.5±0.0^{a}	1907±16^{bc}	931±6^{d}	210±8^{fg}	1186±17^{bcd}
F-019	77.6±0.1^{a}	1877±26^{c}	920±8^{d}	210±8^{fg}	1167±10^{cdef}

*Mean values of two measurements; values followed by the same letters in the same column are not significantly different ($p < 0.05$).

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Table 2.4 Pasting properties of waxy sorghum starch (F-019) isolated by three methods at shorter (10min) and longer (15min) grinding times.

Sample	Pasting temperature	Peak viscosity	Breakdown viscosity	Setback viscosity	Final viscosity
	°C	(cP)	(cP)	(cP)	(cP)
	Enzymatic treatment of decorticated sorghum				
F-019	77.3±0.3 a	2006±4 a	1129±4 a	328±6 a	1205±6 a
F-019*	77.3±0.4 a	2021±10 a	1114±14 a	299±13 bc	1206±8 a
	Enzymatic treatment of whole sorghum				
F-019	77.7±0.1 a	1992±6 a	1123±2 a	314±4 ab	1178±8 b
F-019*	77.3±0.3 a	2018±10 a	1100±10 a	282±11 c	1200±9 ab
	Wet milling under 50°C				
F-019	77.5±0.0 a	1910±11 b	970±12 b	195±1 d	1135±0 c
F-019*	77.5±0.0 a	1914±20 b	944±19 b	182±7 d	1153±8 c

*Samples marked with an asterisk (*) indicate starch isolated using a longer grinding time (15min), while unmarked samples refer to starch isolated using a shorter grinding time (10min).

*Mean values of two measurements; values followed by the same letters in the same column are not significantly different ($p < 0.05$).

Table 2.5 Gelatinization properties of waxy sorghum starches isolated by three methods.

Sample	Gelatinization endotherm				Enthalpy
	To (°C)	Tp(°C)	Tc(°C)	Tc-To (°C)	ΔH(J/g)
	Enzymatic treatment of decorticated sorghum				
F-032	73.7±0.2^{b}	78.3±0.1^{a}	91.3±0.4^{abc}	17.5±0.6^{ab}	21.8±0.1^{a}
F-037	73.9±0.2^{b}	78.5±0.1^{a}	91.4±0.1^{ab}	17.5±0.3^{ab}	21.6±0.1^{a}
F-019	72.2±0.3^{d}	76.8±0.4^{b}	89.9±0.2^{d}	17.6±0.5^{ab}	20.8±0.1^{c}
	Enzymatic treatment of whole sorghum				
F-032	74.1±0.0^{ab}	78.9±0.2^{a}	92.0±0.1^{a}	17.9±0.1^{ab}	21.7±0.1^{a}
F-037	74.5±0.0^{a}	78.7±0.0^{a}	91.3±0.2^{abc}	16.7±0.2^{b}	21.5±0.1^{a}
F-019	73.1±0.0^{c}	77.3±0.1^{b}	90.4±0.3^{cd}	17.3±0.3^{ab}	20.7±0.0^{c}
	Wet milling				
F-032	73.8±0.2^{b}	78.9±0.2^{a}	91.7±0.4^{ab}	17.9±0.2^{ab}	21.1±0.1^{b}
F-037	74.0±0.2^{b}	78.9±0.0^{a}	91.4±0.1^{ab}	17.5±0.3^{ab}	20.7±0.1^{c}
F-019	72.5±0.2^{d}	77.3±0.2^{b}	91.0±0.1^{bc}	18.5±0.3^{a}	20.2±0.2^{d}

*To, Tp, and Tc represent onset, peak, and conclusion gelatinization temperatures, respectively. Tc–To is the gelatinization temperature range (the difference between Tc and To); ΔH, gelatinization enthalpies.

*Mean values of two measurements; values followed by the same letters in the same column are not significantly different ($p < 0.05$).

Table 2.6 Summary of three starch isolation methods for waxy sorghum grains.

Aspect	Wet milling	Enzymatic treatment of decorticated sorghum	Enzymatic treatment of whole sorghum
Purity	Desired purity (ash, protein, damaged starch contents below 0.180%, 0.40% and 1.40%, respectively)		
Recovery	High starch recovery, ranging from 86.6-88.3% (no statistically significant differences among the three methods)		
Color	High quality of starch (higher L*, lower b*)	High quality of starch (higher L*, lower b*)	Undesirable and dark appearance
Functional properties	1. Lower peak, breakdown, and final viscosities 2. Lower gelatinization enthalpy	1. Higher peak, breakdown, and final viscosities 2. Higher gelatinization enthalpy	1. Higher peak, breakdown, and final viscosities 2. lower onset temperature and higher gelatinization enthalpy
Technological Complexity	1. Plate mill for fine grinding 2. Customized starch table to separate starch from protein 3. Pump for pumping starch slurry onto the starch table	1. Decorticator for debranning sorghum grains 2. Alkaline protease for protein hydrolysis 3. pH controller for monitoring pH during protein hydrolysis	1. Alkaline protease for protein hydrolysis 2. pH controller for monitoring pH during protein hydrolysis
Efficiency	1. Extensive time and high-water consumption 2. Limited daily starch isolation due to the starch table	Potentially faster process with lower water usage	Potentially faster process with lower water usage
By-products	Sorghum protein, bran and fiber	Sorghum bran and fiber	Sorghum bran and fiber

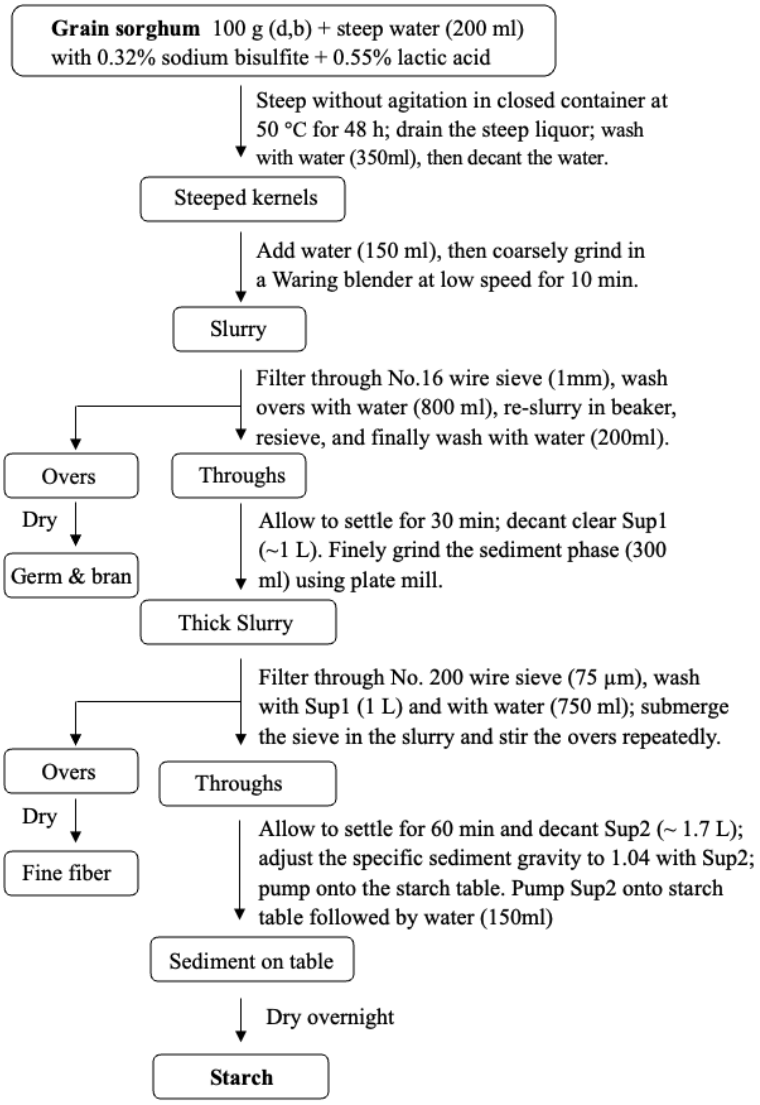

Figure 2.1 The procedure for isolating starch from whole sorghum grains by traditional wet milling method (adapted from Xie and Seib, 2000).

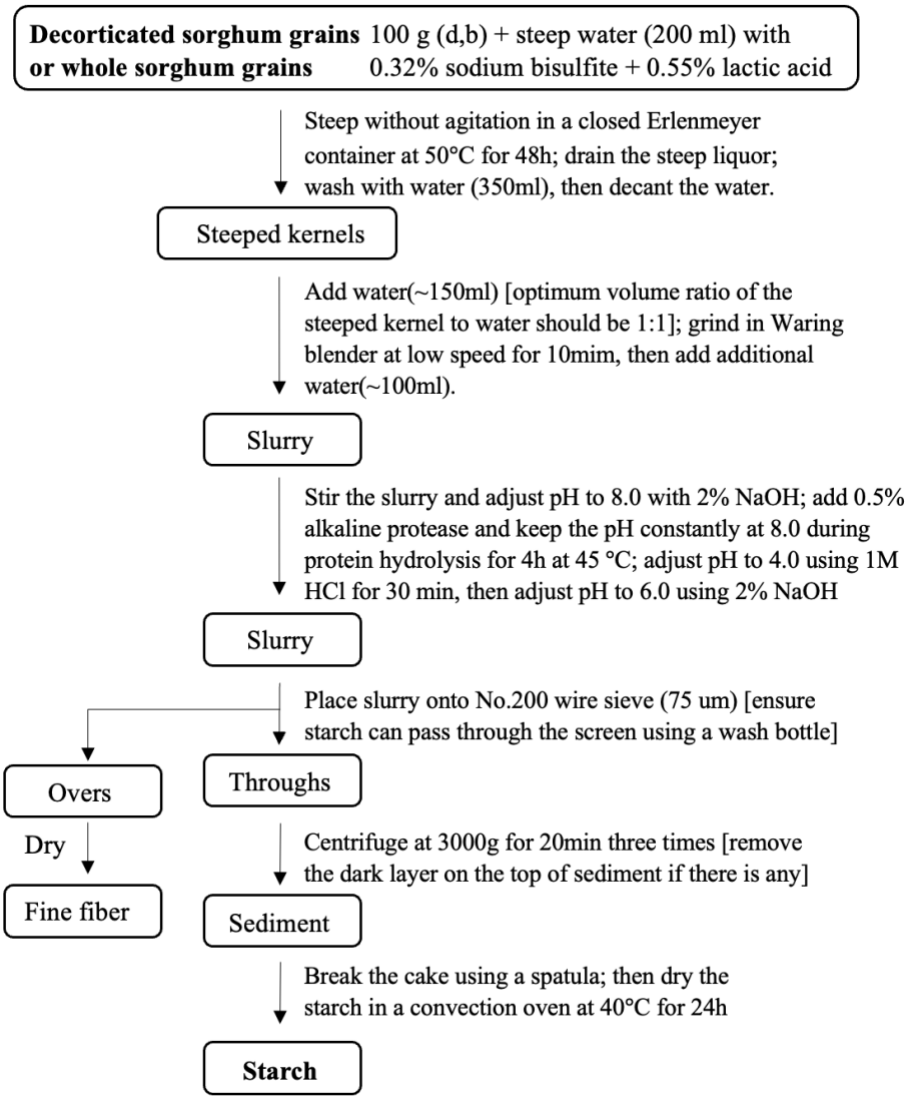

Figure 2.2 The procedure for isolating starch from decorticated sorghum or whole sorghum grains by enzymatic treatment method.

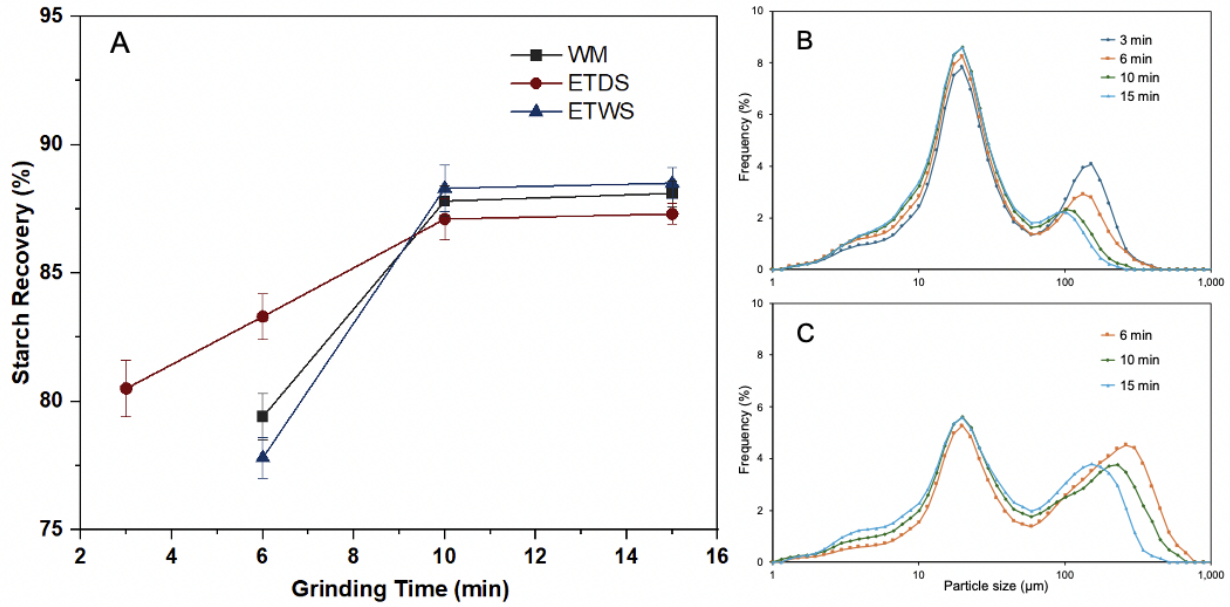

Figure 2.3 Effect of grinding time on the recovery of waxy sorghum starch (F-019) isolated by three methods at low speed (A) (WM: wet milling, ETDS: enzymatic treatment of decorticated sorghum, ETWS: enzymatic treatment of whole sorghum); Effect of grinding time on the particle size distribution of slurry from decorticated sorghum grains (B) and whole sorghum grains (C).

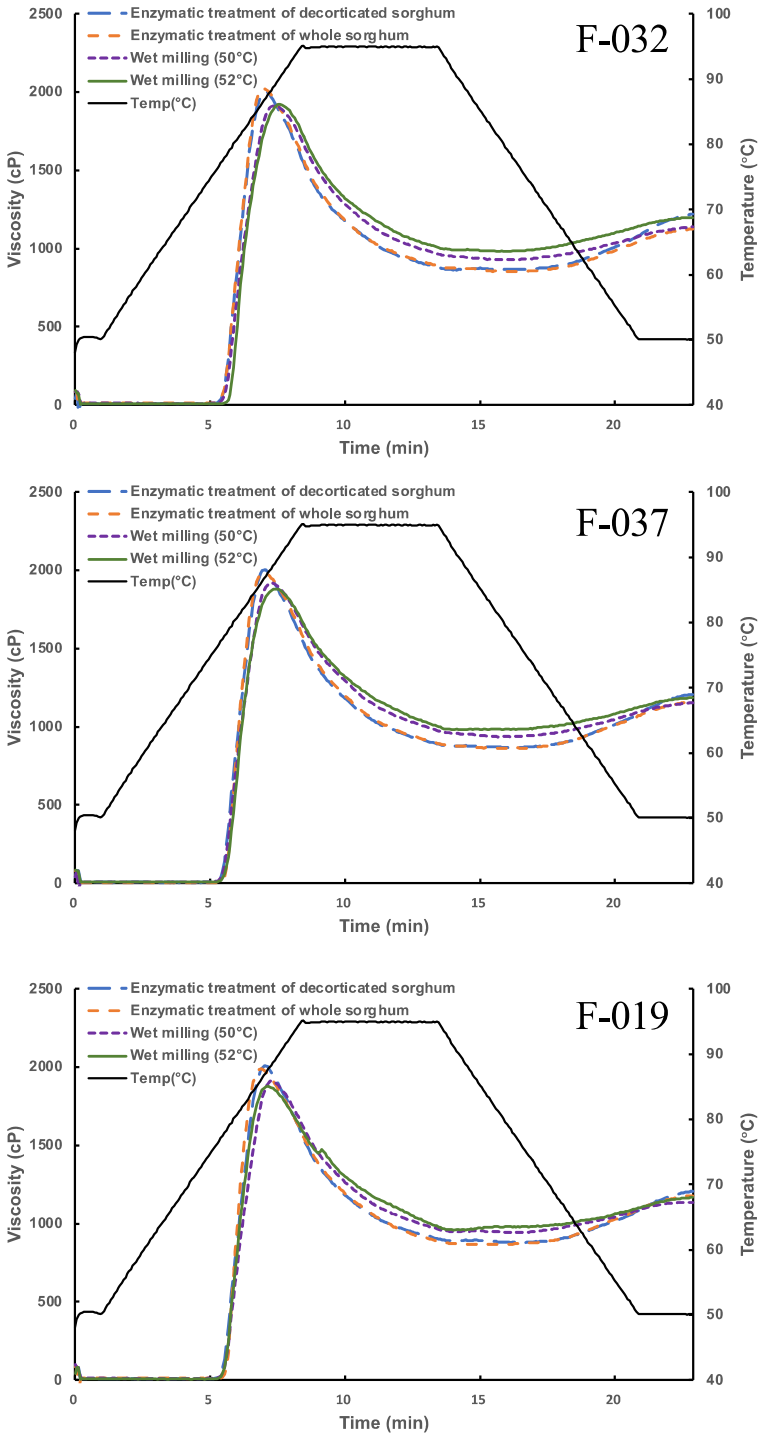

Figure 2.4 Pasting curves of sorghum starches (F-032, F-037, F-019) measured by Rapid Visco-Analyzer (6%), including the wet-milling methods under two steeping temperatures (50°C and 52°C), respectively.

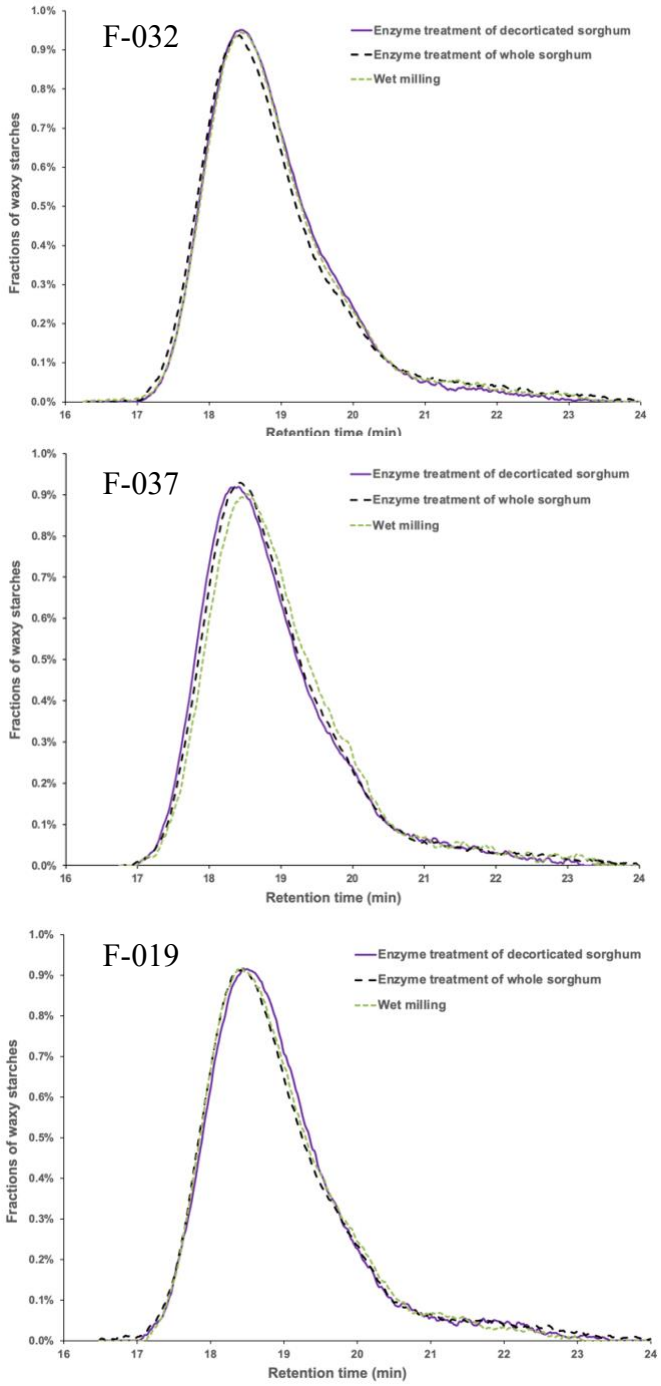

Figure 2.5 Comparison of molecular size distribution of waxy sorghum starches isolated by three different methods.

Chapter 3 - Identifying the Potential of Waxy Sorghum Starches and Flours as Thickeners in Comparison with Waxy Maize Starch

Abstract

A comprehensive study was conducted to evaluate the potential of waxy sorghum starches and flours as food thickeners by examining their structural and functional properties in comparison to waxy maize starch. The specific objectives were to identify waxy sorghum starch and flour with greater thickening power and cold storage stability, explore the relationship between pasting properties and morphology of waxy starch cooks, determine how the retrogradation properties are affected by chain-length distribution, and investigate correlations between the starch content, starch structural features, and functional properties of waxy sorghum starches and flours. Waxy sorghum starches exhibited a greater degree of granule swelling and significantly higher pasting viscosities compared to waxy maize starch. Starches with lower retrogradation tendency contained a higher proportion of chains with DP 6-11 and a lower proportion of chains with DP 12-24. The peak, setback, and final viscosities of waxy sorghum flours were significantly positively correlated with the corresponding viscosities of their starches (peak: $r = 0.606^*$; setback: $r = 0.635^*$; final: $r = 0.656^*$, $p < 0.05$). The total starch content was only found to be significantly correlated with setback ($r=0.694^*$) and final viscosities ($r=0.682^*$, $p<0.05$). In contrast, grain hardness exhibited a significant negative correlation with the peak ($r =-0.785^*$, $p<0.05$) and breakdown ($r =-0.780^{**}$, $p<0.01$) viscosities of waxy sorghum flours. These findings provide valuable insights for sorghum breeding programs and support the commercialization of waxy sorghum grains as food thickeners.

Introduction

Sorghum is the 5th most widely cultivated cereal crop globally, following maize, rice, wheat, and barley, and is known for its desirable agronomic traits, such as heat tolerance, drought resistance and low production costs (Bean et al., 2011, 2019; Zhu, 2014). The United States is the largest producer of sorghum, accounting for over 14% of global production, followed by Nigeria, India, Mexico, Brazil (U.S. Department of Agriculture, 2024). Sorghum has been consumed as a primary food source in Asia, Africa, and South America for many years (Boudries et al., 2009; Dicko et al., 2006; Ramatoulaye et al., 2016). However, it remains underutilized in many developed countries, where it is primarily used for animal feed, bioethanol production, and industrial purposes (Ai et al., 2011; Bean et al., 2019; Ramatoulaye et al., 2016). As a gluten-free grain, sorghum serves as a promising alternative flour for individuals with celiac disease and gluten intolerance (Ciacci et al., 2007; Pineli et al., 2015). Its unique nutritional properties and health benefits, including high dietary fiber, antioxidants, anticancer, and anti-inflammatory properties, make the grain suitable for developing functional foods and nutraceuticals (Abah et al., 2020; Awika & Rooney, 2004; Bean et al., 2019; Espitia-Hernández et al., 2022; Lee et al., 2020). As a result, sorghum has been increasingly utilized in a wide range of food products, such as fermented beverages, flatbreads, noodles, cookies, tortillas, and porridges (Khoddami et al., 2023; Pineli et al., 2015; Taylor & Anyango, 2011b).

Starch, the major storage carbohydrate in sorghum grains, constitutes up to 80% of the dry grain weight (Liu et al., 2012; Zhu, 2014). The utilization and quality of sorghum-based products are largely determined by the properties of its starch. Many important physicochemical and nutritional properties of starch are affected by the ratio of amylose and amylopectin, the two major polymers in the starch granule, as well as the structure of amylopectin (Bean et al., 2019;

Fredriksson et al., 1998; Hsieh et al., 2019a; Sang et al., 2008). Most of the sorghum genotypes contain amylose levels ranging from 20% to 30%, which is typical for normal cereal grains (Zhu, 2014). However, waxy sorghum essentially contains amylopectin in its endosperm starch and has special starch properties that could be used as value-added food ingredients, potentially enhancing its economic value (Hsieh et al., 2019). Waxy starches typically exhibit more extensive swelling when heated in water and give a higher pasting viscosity compared to their normal counterparts (Fredriksson et al., 1998; Hsieh et al., 2019a). The potential starch-related uses of waxy sorghum are diverse, including foods and alcoholic beverages, thickening agent, packaging materials, substrate for bacteria, and biofuel production (Ai et al., 2011; Kang et al., 2022; Kwon et al., 2005; McGinnis & Webster, 2024; Pineli et al., 2015; Šárka & Dvořáček, 2017; Soe Htet et al., 2022; Taylor & Anyango, 2011b, 2011a).

Clean label is an emerging global trend in packaged food, requiring food products to be made with natural ingredients and minimal processing (Park & Kim, 2021). However, the application of unmodified starches in commercial products is limited due to their undesirable functional properties, such as thermal instability and a high tendency to retrograde (Hsieh et al., 2019a, 2019b; Wang et al., 2018). Thus, starches are often chemically modified to overcome one or more shortcomings of unmodified starches. Currently, waxy maize starch is the preferred base starch, which is further processed and modified to produce food thickeners (Hsieh et al., 2019a, 2019b; Wang et al., 2018). Waxy sorghum has the potential to be used directly as a food thickener without chemical modification, but we do not know how starches properties in different waxy sorghum grains are compared with those of waxy maize starch. In addition, waxy sorghum flours, which are non-GMO and gluten-free, would be considered clean-label functional

flours and particularly useful in all-natural foods where chemically modified ingredients are banned.

In this study, we conducted a comprehensive analysis of the fine structures of amylopectin, morphology of starch granules during cooking, pasting and thermal properties of waxy sorghum samples, and compared their properties with waxy maize starch to evaluate their unique attributes as food ingredients. The specific objectives were to identify waxy sorghum starches and flours with higher thickening power and greater freezing-thawing stabilities, examine how the pasting and retrogradation properties of starch are affected by the structural properties (chain-length distribution and molecular distribution), and investigate the correlations between starch structural features, functional properties of starches and flours, and their compositional characteristics. Additionally, a rapid method was developed to differentiate between waxy and normal sorghum grains, with potential applicability to other cereal crops. This research could provide valuable information for sorghum breeding programs and promote the commercialization of waxy sorghum grains as food thickeners.

Materials and methods

Materials

Thirty-five waxy grain sorghum samples were obtained from the Center for Sorghum Improvement at Kansas State University (Table 3.1). The samples include twelve waxy sorghum grains grown in Assaria, Kansas in 2021, twelve of these same samples from Colby, KS, and eleven of these same samples from Sunray, TX. Two commercial sorghum samples were supplied by NuLife Market (Scott City, KS), including waxy burgundy whole grain sorghum and white whole grain sorghum. In addition, commercial waxy maize starch (Amioca) was obtained

from Ingredion Inc. (Bridgewater, NJ). Waxy wheat (Mattern) kernels were provided by Dr. Robert Graybosch (USDA-ARS, Lincoln, NE, USA), and normal wheat kernels were purchased from MKC (Manhattan, KS) in 2023. Each sorghum sample was hand-cleaned to remove foreign materials and then stored at room temperature. Danisco FoodPro alkaline protease, with a specific activity ranging from 580,000 to 650,000 DU/g, was obtained from DuPont Nutrition and Health (Rochester, New York, USA). Isoamylase (E-ISAMY; from Pseudomonas sp.) was purchased from Megazyme International Ltd. (Co. Wicklow, Ireland). All other chemicals were analytical grade.

Methods

Starch isolation

Waxy sorghum starch was isolated using an enzymatic treatment method based on the procedure described by Huang & Shi (2024, unpublished). Sorghum grains were decorticated to 20% by weight using a tangential abrasive dehulling device (Venables Machine Works, Saskatoon, SK, Canada). The decorticated grains (100 g, dry basis) were steeped in 200 mL of distilled water containing 0.32% sodium bisulfite and 0.55% lactic acid at 50°C for 48 hours. After steeping, the kernels were rinsed with 350 mL of distilled water and subsequently ground with 150 mL of distilled water in a Waring blender (Quaker City, model No. 4E, Straub Co., Warminster, PA) at low speed for 10 minutes. The resulting slurry was adjusted to pH 8.0 with a 2.0% NaOH solution, followed by the addition of 0.5% alkaline protease (w/w). The hydrolysis process was conducted at 45°C for 4 hours, with the pH maintained at a constant level of 8.0 through continuous monitoring. After hydrolysis, the protease was deactivated by adjusting the pH to 4.0 with 1M HCl and holding for 30 minutes. The pH was then readjusted to 6.0 with a 2.0% NaOH solution. The slurry was centrifuged three times with distilled water at 3000 × g for

20 minutes each, and any dark layer on top of the sediments was carefully removed. The sediment (starch) was collected and dried in a convection oven at 40 °C overnight.

Iodine staining of sorghum grains and starches

Sorghum kernels were cut using a stainless-steel scalpel, and the exposed surfaces were stained with 0.1N iodine solution (1.9 g of potassium iodide and 0.64 g of iodine crystals in 50 mL of distilled water). Waxy and normal wheat kernels were also stained for comparison with waxy sorghum kernels. After staining, the fractured kernels were observed under a digital microscope VHX-7000N (Keyence Corporation, Osaka, Japan). In addition, iodine staining was applied to isolated sorghum starches to quantify the contamination level of normal sorghum starch in waxy sorghum starch. Sorghum starch (0.01 g) was suspended in 0.5 mL of distilled water and mixed with 20 µL of 0.1N iodine solution (Hsieh et al., 2019a). After vortex mixing for 5 s, 20 µL of the stained suspension was transferred onto a microscope slide, covered with a coverslip, and immediately observed under a light microscope (Olympus BX51, Olympus America, Inc., Melville, NY) using a 40X objectives lens. To optimize staining conditions, lower iodine concentrations (0.05N and 0.025N) were also evaluated. Each sample was stained in duplicate, and three random sets of images were captured during each staining session. The total number of red and blue granules of three random sets in one staining session were counted to calculate the contamination of normal sorghum starch in waxy sorghum starch.

Calculation:

$$\% \text{ Normal Starch (contamination)} = \frac{\text{Total Purple Granules} * 100}{\text{Total Purple Granules} + \text{Total Red Granules}}$$

(1) Normal starch granules were stained dark blue, whereas waxy starch granules were stained reddish brown.

Morphology of cooked starch granules

Starch (0.03 g) was added to distilled water (3 mL) and mixed using a magnetic stirrer in a water bath at temperatures of 25, 60, 70, 80, and 90°C for 20 minutes, following the method of Wang et al. (2018). To highlight the morphological differences between cooked waxy maize and waxy sorghum starches, the cooked starch slurry (1%, w/w) was stained with 120 µL of 0.1N iodine solution after cooling. After vortex mixing for 5 s, 20 µL of the stained slurry was placed on a microscope slide, covered with a coverslip, and observed under a light microscope (Olympus BX51, Olympus America, Inc., Melville, NY) using a 40X objectives lens.

Preparation of waxy sorghum flour

Whole sorghum grains were decorticated using a tangential abrasive dehulling device (Venables Machine Works, Saskatoon, SK, Canada) equipped with an 80-grit abrasive disk, removing approximately 20% of the bran and germ by weight, as described by Park et al. (2006). The debranned sorghum grains were subsequently ground using a Udy mill with a 1.0-mm screen. The particle size distribution of waxy sorghum flours and commercial white pearled grain sorghum flour was determined using a Beckman/Coulter LS 13 320 laser diffraction particle size analyzer (Beckman/Coulter Particle Characterization, Miami, FL) (Xu et al., 2022).

Moisture, ash, protein, and total starch content

The moisture content of waxy sorghum starch and flour was determined using AACC Approved Method 44-15A, while the ash content was measured according to AACC Approved Methods 08-01 (AACC 2000). Protein content was assayed by a nitrogen determinator (LECO FP-828, St. Joseph, ML). The total starch content of waxy sorghum flour was quantified using a Megazyme Total Starch kit (Wicklow, Ireland) according to AACC Approved Methods 76-12.

X-ray diffractometry and relative crystallinity

The moisture content of waxy sorghum and maize starch samples was adjusted to approximately 20% in a sealed desiccator containing water at the bottom at room temperature prior to analysis. The equilibrated starch samples were then evenly packed into an XRD sample holder. The crystallinity pattern and relative crystallinity of the samples were analyzed using a wide-angle X-ray diffractometer (Bruker AXS D8 Advance, Karlsruhe, Germany) operated at 40 kV and 40 mA, with diffraction angle (2θ) ranged from 3° to 35° and a step size of 0.05° (2θ). The XRD patterns were recorded using a copper anode with a fixed K-alpha wavelength of 1.5419 Å. Relative crystallinity was estimated by calculating the ratio of the crystalline peak areas to the total area of the diffractogram (Komiya & Nara, 1986) using OriginPro 2023b software (Northampton, MA, USA).

Molecular distribution of debranched waxy starches

The starch sample (4-5 mg) was mixed with 1.75 ml of water in a 15 ml centrifuge tube and heated in a boiling water bath for 20 minutes with gentle stirring. After cooling to room temperature, 250 µL of sodium acetate buffer (pH 4.4) and 2 µL of isoamylase (Megazyme, Wicklow, Ireland) were added, and the mixture was incubated at 50 °C for 12 h to allow starch debranching. The enzyme was then inactivated by heating the mixture in a boiling water bath for 10 minutes with mild stirring. After debranching, 13 ml of ethanol was added to precipitate the starch, followed by placing the mixture in a freezer for 1 h. The precipitated starch was collected by centrifugation at 3000×g for 20 minutes and vacuum dried. The dried, debranched starch sample (≈4 mg) was dissolved in 4 mL of dimethyl sulfoxide (DMSO) containing 0.5% (w/w) lithium bromide (LiBr) (Vilaplana & Gilbert, 2010) in a boiling water bath overnight with gentle stirring. After cooling to room temperature, the solution was filtered through a 0.45 µm filter for

analysis by gel permeation chromatography (GPC) (PL-GPC 220, Polymer Laboratories Varian, Amherst, MA, USA) equipped with a refractive index (RI) detector, based on the method of Shi et al. (2018). Data were processed using Cirrus™ GPC software version 3.0 (Agilent Technologies, Santa Clara, CA, USA).

Chain-length distribution of debranched waxy starches

The debranched starch was dissolved in 0.01 M acetate buffer (pH 4) at a concentration of 2 mg/ml, filtered through a 0.45 μm filter, and analyzed using a high-performance anion-exchange chromatography system (HPAEC, Dionex ICS-3000, Dionex Corp., Sunnyvale, CA, USA) equipped with a pulsed amperometric detector and a CarboPac™ PA1 analytical column. The chain-length distribution of amylopectin was measured based on the method of Cai & Shi (2010). The eluents A and B were 150 mM sodium hydroxide and 150 mM sodium hydroxide containing 500 mM sodium acetate, respectively. A gradient program was applied as follows: 40% of eluent B at 0 min, 50% at 2 min, 60% at 10 min, and 80% at 40 min. The flow rate was set at 1 mL/min, with a constant separation temperature of 25 °C.

Thermal properties of waxy sorghum starches and flours

The gelatinization and retrogradation properties of waxy sorghum starches and flours were measured by differential scanning calorimetry (DSC) as described by Shi & Seib (1992). Starch or flour samples (10mg) were accurately weighed into stainless-steel pans, mixed with distilled water at sample to water ratio of 1:1.5 (corrected by sample moisture), and sealed. An empty pan was used as a reference. Gelatinization properties of starch and flour samples were determined by heating the samples from 10 °C to 120 °C at the rate of 10 °C/min using a Q200 DSC (TA Instrument, New Castle, DE, USA). For retrogradation analysis, the gelatinized sample in pans were stored at 4 °C for 7 days before being reheated under the same conditions. The onset (To),

peak (Tp), and conclusion (Tc) temperatures, as well as the endothermic enthalpy (ΔH), were calculated from the DSC thermograms using Universal Analysis software (TA Instrument, New Castle, DE, USA).

Pasting properties of waxy sorghum starches and flours

The pasting properties of waxy sorghum starches and flours were determined using a Rapid Visco Analyzer (RVA-4500, Perten Instruments, Sweden). A 6% starch suspension or an 8% flour suspension was prepared by mixing 1.68g (dry basis) of starch with 26.32 g of distilled water (corrected by sample moisture), or 2.24 g of flour with 25.76 g of distilled water, respectively. The measurements were performed by following the STD2 temperature profile (Approved Method 76-21.02, Cereals & Grains Association, 2009), where the sample was held at 50 °C for 1 min, heated to 95 °C for 7.5 min, held at 95 °C for 5 min, cooled to 50 °C for 7.5 min, and held at 50 °C for 2 min. The paddle rotation speed was set at 160 rpm. The values of pasting temperature, peak viscosity, breakdown viscosity, setback viscosity, and final viscosity were recorded.

Statistical analysis

All experiments were performed in duplicate. The collected data were analyzed using IBM SPSS Statistics Version 26 (IBM Corporation, New York, USA), with Pearson correlation analysis conducted to assess relationships between variables. Analysis of variance (ANOVA) was applied to evaluate whether the data exhibited statistically significant differences ($p < 0.05$). Correlations with probability values of $p < 0.01$ and $p < 0.05$ were considered statistically very significant and significant, respectively. The results were expressed as means ± standard deviations (SD).

Results and discussion

Iodine staining of sorghum grains and isolated starches

Iodine staining could effectively differentiate between waxy and normal (amylose-containing) sorghum based on their distinct color reactions with iodine (Zhu, 2014). The fractured waxy sorghum kernels stained reddish-brown, while the normal sorghum kernels stained dark blue due to the complex formation between iodine and the linear structure of amylose (Madhu et al., 2016) (Figure 3.1). The staining of sorghum endosperm was non-uniform, with the floury region exhibiting a deeper color and the vitreous region appearing lighter. However, both normal and waxy wheat kernels exhibited uniform staining (Figure 3.1). The non-uniformity of staining was likely attributed to the physical structure of the sorghum endosperm. In the vitreous endosperm, starch granules are tightly packed with protein bodies and surrounded by a continuous protein matrix, whereas in the floury endosperm, they are more loosely packed within a discontinuous protein matrix (Bean et al., 2011, 2019; Hoseney et al., 1974). Consequently, the loosely packed structure of the floury endosperm in sorghum may facilitate greater iodine penetration, resulting in more intense staining compared to the vitreous endosperm.

Iodine staining can also effectively differentiate between waxy and normal starch granules based on their distinct color reactions with iodine (Brust et al., 2020). Most sorghum samples from Colby and Sunray cannot be classified as waxy sorghum due to the contamination level of normal starch exceeding 5.0% (Table 3.1). These samples, cultivated in small breeding plots, may have been cross-pollinated with pollen from nearby non-waxy varieties, leading to increased contamination levels. Figures 2 and 3 show the variation in iodine staining color of normal and waxy sorghum starches (with 1.9% contamination) under different iodine concentrations (0.1N,

0.05N, and 0.025N). Tester et al. (2004) reported that higher iodine concentration increased the availability of iodine molecules, enhancing their binding affinity to starch granules and resulting in a more intense color reaction. Both 0.1N and 0.05N iodine concentrations provided a clear and strong visual distinction between waxy and normal sorghum starch granules, allowing for the assessment of contamination of normal sorghum in waxy sorghum starch, whereas 0.025N iodine did not provide clear color differentiation.

Hardness of sorghum grains, particle size, and chemical composition of sorghum flours and starches

Ten waxy sorghum samples were selected from thirty-five waxy sorghum samples, including seven from Assaria, KS, two from Colby, KS, and one from Sunray, TX, based on their contamination of normal sorghum starch being below 5% (Table 3.1). The selected sorghum grain samples exhibited a range of endosperm hardness, as indicated by single kernel characterization system (SKCS) hardness indices varying from 65.8 to 87.8. Their average flour particle size was between 65.6 and 82.0 μm, comparable to that of commercial pearled white sorghum flour (84.27 μm). The total starch content of decorticated waxy sorghum flours ranged from 81.8% to 84.7%, while their protein and ash contents varied from 10.40% to 11.69% and 0.855% to 1.163%, respectively. The recovery and purity of starch isolated from decorticated waxy sorghum grains are also summarized in Table 3.3. All isolated starches achieved the desired purity, with ash and protein contents below 0.180% and 0.40%, respectively.

X-ray diffraction (XRD) and relative crystallinity

Waxy starch granules from sorghum and maize presented a typical A-type crystalline pattern, with main peaks identified at 2θ values of 15°, 17°, 18°, and 23° in the X-ray diffractogram (Figure 3.5). The A-type crystalline structure is characterized by a double helix

formed by amylopectin molecules arranged in a monocycle configuration, which is also found in other cereal starches (Alves et al., 2014; Cheetham & Tao, 1998). The relative crystallinity of waxy sorghum starches (35.9% to 40.3%) was found to be higher than that of waxy maize starch (34.5%). Among waxy sorghum starches, F-032, F-037, F-004, F-025, and F-041 exhibited a greater percentage of relative crystallinity, ranging from 38.0% to 40.3%, compared to F-010, F-019, F-065, F-067, F-236, and waxy burgundy sorghum starch, which showed values between 35.9% and 37.8%. Chung et al. (2011) reported that waxy starch with a large proportion of long chains formed stable and highly crystalline structures.

Molecular size distribution of debranched starches

The molecular size distribution of debranched waxy starches, expressed as hydrodynamic radius, (R_h), is presented in Figure 3.6. Both waxy sorghum and maize starches displayed a bimodal distribution, as shown in Peaks 1 (left) and 2 (right). The fractions corresponding to peaks 1 and 2 in the GPC profiles were composed of debranched amylopectin. Peak 1 primarily contained short branch chains, including A and short B chains (A+B1 chains), whereas peak 2 was composed of long B chains (Hsieh et al., 2019a; Wu & Gilbert, 2010). Normal sorghum starch exhibited the highest proportion of the high-molecular size fraction (Peak 3), attributed to debranched amylose molecules. Notably, waxy maize starch had a higher proportion of the high-molecular size fraction (Peak 2) than waxy sorghum starches, while waxy sorghum starches exhibited a higher Peak1/Peak2 ratio (Figure 3.7). Among waxy sorghum starches, F-037, F-067, and F-236 exhibited the highest ratio of Peak 1 to Peak 2, whereas F-025 had the lowest ratio of Peak 1 to Peak 2. The lower ratio observed in this study was found to be related to lower pasting peak and breakdown viscosities (Figure 3.8 and Table 3.11). Some studies suggested that a higher proportion of Peak 2 might be attributed to increased growth temperature, which

decreases the activities of starch synthase and starch branching enzymes, thus limiting the formation of short amylopectin chains and decreasing the branching degree (Fan et al., 2019; Gu et al., 2022).

Chain-length distribution of waxy starches

The chain-length distribution and relative peak areas (%) of waxy sorghum and maize starches are shown in Table 3.4. The maximum chain length that could be measured ranged from 58-61, while DP 6 appeared to be the minimum detectable size, which agreed with the observation by Hsieh et al. (2019). Both waxy sorghum and waxy maize starches displayed a shoulder at DP 18-21 and the maximum proportion of chains at DP 14. These findings are consistent with previous studies that starches from various botanical sources typically exhibit a shoulder at DP 18-21 (Hanashiro et al., 1996; Hsieh et al., 2019a; Jane et al., 1999). The chain-length distribution of amylopectin can be fractioned into DP 6-12, DP 13-24, DP 25-36, and DP >37, representing A chains, B1 chains, B2 chains, and B3 chains, respectively, based on the amylopectin cluster model proposed by Hizukuri (1986). The crystalline regions of starch granules are predominantly composed of amylopectin branch chains with DP 6-24, where chains with DP 13-24 could form double helices that are packed into crystalline clusters (Hanashiro et al., 1996; Srichuwong et al., 2005). Some studies reported that the retrogradation of waxy starches is inversely related to the proportion of short chains with DP 6-11, especially those with DP 6-9 (Hsieh et al., 2019a; Shi & Seib, 1992, 1995). In this study, the levels of unit chains with DP 6-9 as well as DP 6-11 were included due to their significant roles in influencing the retrogradation of waxy starches. Waxy sorghum starches had a lower proportion of chains with DP 6-9 (≤6.56%) and DP 6-11 (≤15.99%) but a higher proportion of chains with DP 12-24 (≥59.21%). In contrast, waxy maize starch exhibited a higher proportion of chains with DP 6-9

(7.32%) and DP 6-11 (16.67%) but a lower proportion with DP 12-24 (57.08%). Among the waxy sorghum starches, F-032 exhibited a significantly lower proportion of chains with DP 6-11, while F-037, F-025, and F-236 had lower proportion of chains with DP 12-24.

Pasting properties

Pasting properties and morphology of waxy starches

The pasting profiles and corresponding attributes of waxy sorghum and waxy maize starches are shown in Figure 3.7A and Table 3.4. In general, compared to amylose-containing starches, waxy starches typically exhibit higher peak viscosities followed by a more significant breakdown and a lower degree of setback after pasting (Abdel-Aal et al., 2002; Hung et al., 2007; Zeng et al., 1997). In this study, waxy sorghum starches exhibited lower pasting temperatures (≤78.7 °C), and higher peak viscosities (≥1647 cP) compared to normal sorghum starch (91.9 °C, 599 cP). The difference is likely due to amylose inhibiting the swelling of starch granules by forming complexes with lipids, resulting in reduced peak viscosity and higher pasting temperatures (Sang et al., 2008). The amylose-lipid complex increased the rigidity of starch granules by inhibiting swelling, resulting in the lower breakdown for normal sorghum starch (159 cP) compared to waxy sorghum starches (≥953 cP). Additionally, normal sorghum starch exhibited a higher setback than waxy sorghum starches due to its higher amylose content. The linear amylose molecules can more readily re-align and re-associate into ordered structures during the cooling stage, leading to restricted water mobility and increased viscosity (Ao & Jane, 2007).

Changes in the fine structure of waxy starch, such as chain-length distribution, degree of branching, granule crystallinity, and molecular size, could significantly affect its functional properties (Lin et al., 2016). Waxy sorghum starches exhibited higher pasting temperatures

(≥77.1 °C) compared to waxy maize starch (72.6 °C), indicating their greater resistance to swelling, rupture, and loss of molecular order. Some studies reported that starches with higher pasting temperature had stronger binding forces within the granule interior, forming a stronger double helical structure that required more energy to disrupt during pasting (Kumar & Khatkar, 2017; Peroni et al., 2006). Waxy sorghum starches also exhibited higher peak, breakdown, setback, and final viscosities compared to waxy maize starch. Among the waxy sorghum starches, F-037, F-019, and F-041 showed higher peak, breakdown, and final viscosities compared to other sorghum varieties (Table 3.5). F-010, F-004, F-025, and waxy burgundy sorghum starch displayed significantly lower peak and final viscosities. While Hung et al. (2007) suggested that higher pasting viscosities are typically associated with increased relative crystallinity, our study did not observe a clear correlation between pasting peak viscosities and relative crystallinity.

The microscopic observations in starch cooks are in accordance with the results of pasting properties (Figure 3.4). The images revealed that all starch granules increased in size as the temperature rose from 25 °C to 80 °C, with waxy maize starch swelling more prominently than waxy sorghum starches. When the cooking temperature was increased to 90 °C, waxy maize starches lost their granular structure and broke into fragments. In contrast, waxy sorghum starches continued to swell while largely retaining their granular integrity. Wang et al. (2018) reported that the pasting properties of starch are influenced by the volume of the swollen granules, their deformability (stiffness), and attractive forces between the swollen granules. The results provide visual evidence that the higher peak viscosity of waxy sorghum starches, compared to waxy maize starch, is due to the ability of sorghum starch granules to swell more extensively without losing their granular structure.

Pasting properties of waxy sorghum flours

The pasting curves and the corresponding attributes of waxy sorghum flours, along with waxy maize starch as a reference (8%, w/w), are shown in Figure 3.7B and Table 3.6. In general, the pasting properties of waxy sorghum flours could be affected by several factors, such as starch concentration, amylopectin branching patterns, the presence of non-starch components, and particle size distribution (Batey & Curtin, 2000; Bean et al., 2011; Chanapamokkhot & Thongngam, 2007; Ragaee & Abdel-Aal, 2006; Zhang & Hamaker, 2005). Despite having a higher starch content (~ 6.5% solids), waxy sorghum flours exhibited significantly lower pasting properties than isolated waxy sorghum starch (~ 6%, Figure 3.7A). The decrease may be attributed to the presence of non-starch components, such as protein matrix, inhibiting the swelling of starch granules by forming a network that physically restricts their expansion during pasting (Hamaker & Bugusu, 2003). Waxy sorghum flours still displayed a higher pasting temperature (≥77.5°C) than waxy maize starch (71.5°C). Among the waxy sorghum flours, F-037 and F-019 exhibited higher peak (≥1423 cP), breakdown (≥571 cP), and final viscosities (≥1567 cP) compared to the other varieties (Table 3.6). In contrast, F-025, F-067, and waxy burgundy sorghum flour had lower peak (≤1261 cP) and breakdown viscosities (≤434 cP). Other waxy sorghum flours, such as F-010, F-004, and F-065, exhibited reduced peak (1306 to 1365 cP) and final viscosities (1349 to 1413 cP). Our study found that pasting peak viscosities of waxy sorghum flours were not correlated with their total starch content. However, they are significantly positively correlated with the peak viscosities ($r=0.606^*$, $p<0.05$) of their corresponding sorghum starches. Grain hardness was significantly negatively correlated with pasting peak viscosities ($r = -0.758^*$, $p < 0.05$), consistent with previous findings suggesting that harder sorghum varieties possess a more continuous and extensive protein matrix surrounding

the starch granules, leading to lower water absorption, less granule swelling, and reduced peak viscosities during pasting (Buffo, 1998; Shull et al., 1990).

Thermal properties

Gelatinization properties of waxy sorghum starches and flours

The gelatinization temperatures and enthalpies of waxy sorghum starches and flours are summarized in Tables 3.7 and 3.8, respectively. The gelatinization enthalpy (ΔH) measures the loss of molecular order in starch, which is related to the degree of crystallinity (Cooke & Gidley, 1992; Singh et al., 2007). Waxy sorghum starches exhibited greater gelatinization enthalpies (ΔH, 20.4-22.2 J/g of starch) and a narrower range of gelatinization temperatures (ΔT, 15.7-19.1°C) compared to waxy maize starch (ΔH, 19.1 J/g of starch; ΔT, 23.9°C). These findings align with the results that waxy sorghum starches possessed higher relative crystallinity than waxy maize starch (Figure 3.5). High gelatinization temperatures of starch are related to stronger crystalline structures and greater molecular order (Campanha & Franco, 2011). Waxy sorghum starches exhibited higher gelatinization temperatures at onset (≥72.7°C), peak (≥77.1°C), and conclusion (≥89.7°C) compared to those of waxy maize starch (65.7°C, 73.7°C, and 89.5°C, respectively). The wide range and the early start of the gelatinization temperature of waxy maize starch suggest that amylopectin chains in the crystalline regions were weakly and loosely organized (Farias et al., 2020; Hsieh et al., 2019a). The crystalline structure within waxy sorghum starch was more ordered and exhibited greater thermal stability. Waxy sorghum starches had a higher proportion of chains with DP 12-24 (Table 3.4) compared to waxy maize starch, likely forming stronger double-helical structures that required more energy to disassociate.

Among all waxy sorghum starches, F-065, F-067, F-236, and waxy burgundy sorghum starch, which exhibited lower relative crystallinity, showed lower gelatinization temperatures (Table 3.7). F-032 and F-041 had higher peak temperatures (≥79.0°C) than other samples, likely due to their better crystallite quality, which is associated with longer and more stable double helices. Waxy sorghum starches, such as F-032, F-037, F-004, and F-025, with higher relative crystallinity, exhibited relatively higher gelatinization enthalpy (ΔH ≥21.8 J/g of starch). It has been proposed that gelatinization enthalpy obtained by DSC measurement is due to the melting of imperfect amylopectin-based crystals, including crystal packing and helix melting enthalpies (Lopez-Rubio et al., 2008). The relative crystallinity of waxy sorghum starches was strongly correlated with the gelatinization enthalpy values ($r=0.942^{**}$, $p<0.01$). Waxy sorghum flours had a broader range of gelatinization temperatures (ΔT, 21.2-24.7°C) but lower enthalpies (ΔH, 12.1-13.1 J/g of flour) compared to waxy sorghum starches, likely due to the presence of non-starch components, such as proteins and lipids. These components may interact with starch granules, restricting their swelling and requiring increased temperature for gelatinization (Alcázar-Alay & Meireles, 2015; Wang et al., 2020). In addition, the cell wall material in the flours may act as a barrier, limiting water movement toward the starch granules and reducing the efficiency of heat transfer (Marshall, 1992).

Retrogradation properties of waxy sorghum starches and flours

The melting temperatures and enthalpies of retrograded waxy sorghum starches and flours, after being stored at 4°C for 7 days, were significantly lower compared to the gelatinization temperatures and enthalpies of native waxy sorghum starches and flours (Tables 3.9 and 3.10). The freezing-thawing stability of starch has been shown to be related to long-term retrogradation, predominantly attributed to the recrystallization of amylopectin (Jane et al., 1999; Shi & Seib,

1992). Waxy sorghum starches exhibited a greater retrogradation tendency compared to waxy maize starch (ΔH, 13.6 J/g of starch; ΔT, 33.5 °C), as indicated by their higher retrogradation enthalpy (ΔH, 14.2-16.5 J/g of starch) and a wider range of retrogradation temperatures (ΔT, 34.0-36.4 °C). Among all waxy sorghum starches, F-032 and F-041 exhibited significantly higher values in retrogradation enthalpies (ΔH ≥15.4 J/g of starch), indicating a stronger retrogradation tendency. The remaining waxy sorghum starches had relatively lower and statistically similar retrogradation enthalpy values (14.2–14.9 J/g of starch). Waxy sorghum flours exhibited a narrower range of melting temperatures (ΔT ≤33.8°C) and lower conclusion temperature (Tc ≤ 71.0°C) compared to their corresponding waxy sorghum starches (Table 3.9). It is likely due to the presence of non-starch components, such as proteins, lipids, and fibers, which could interfere with the recrystallization of amylopectin by restricting molecular mobility and disrupting the formation of stable crystalline structures.

Starch crystallites are predominantly formed by amylopectin branch chains with DP 6–24 (Hizukuri, 1986). Waxy maize starch, which had a slightly lower retrogradation tendency compared to waxy sorghum starches, possessed a higher proportion of chains with DP 6–11 (16.67%) and DP 6–9 (7.32%), but a lower proportion of chains with DP 12–24 (57.08%) (Table 3.4). Among all the samples, retrogradation enthalpy was significantly negatively correlated with the proportion of chains with DP 6–11 ($r=-0.684^*$, $p<0.05$), and positively correlated with the proportion of chains with DP 12-24 ($r=0.678^*$, $p<0.05$). These results are consistent with previous studies that a greater retrogradation tendency is closely related to a lower proportion of branch chains with DP 6-11 and a higher proportion of chains with DP 12-24 (Hsieh et al., 2019a). The external chains (A chains) of amylopectin, especially DP 6-8, might play an important role in retrogradation stability by disrupting the alignment of inter- and intra-molecular

chains, resulting in waxy starches exhibiting significantly lower retrogradation enthalpy (Vamadevan & Bertoft, 2018).

Correlations of chain length distribution of amylopectin, compositional characteristics, pasting properties of starches and flours

The pasting properties of waxy sorghum flours could be influenced by several factors, including chain length distribution of amylopectin, starch concentration, grain hardness (the interaction between starch granules and the surrounding protein matrix), and the presence of non-starch components (Batey & Curtin, 2000; Bean et al., 2011; Chanapamokkhot & Thongngam, 2007; Ragaee & Abdel-Aal, 2006; Zhang & Hamaker, 2005; Zhu, 2014). Understanding these relationships is essential for optimizing the functional properties of sorghum-based products. Pearson correlation analysis was conducted to quantify the strength of associations between these factors (Table 3.11). The chain-length distribution of amylopectin played an important role in determining the functional properties of waxy sorghum starch. The proportion of chains with DP 6-11 exhibited a significant negative correlation with the pasting temperature ($r=-0.701^*$, $p<0.05$), while the proportion of chains with DP 12-24 showed a significant positive correlation ($r=0.705^*$, $p<0.05$). The ratio of peak 1 (short chains) to peak 2 (long chains) was positively correlated with the peak ($r=0.669^*$, $p<0.05$) and breakdown ($r=0.708^*$, $p<0.05$) viscosities of waxy sorghum starches (Figure 3.6). As discussed earlier, retrogradation enthalpy exhibited a significant negative correlation with the proportion of amylopectin chains with DP 6-11 ($r = -0.684$, $p < 0.05$) and a positive correlation with the proportion of chains with DP 12-24 ($r = 0.678$, $p < 0.05$).

The total starch content directly impacts the pasting viscosities in waxy sorghum flours, with higher starch content generally correlating with increased pasting viscosities due to

enhanced water absorption and starch granule swelling during pasting (Bean et al., 2019). However, in our study, the total starch content was only significantly correlated with setback ($r=0.694^*$, $p<0.05$) and final viscosities ($r=0.682^*$, $p<0.05$). This suggests that other factors, such as the pasting of corresponding starch granules and the presence of non-starch components, may also affect their pasting properties. The peak, setback and final viscosities of waxy sorghum flours were significantly positively correlated with the peak ($r=0.606^*$, $p<0.05$), setback ($r=0.635^*$, $p<0.05$), and final ($r=0.656^*$, $p<0.05$) viscosities of their corresponding sorghum starches. No significant correlation was observed between their breakdown viscosities. Grain hardness was significantly negatively correlated with the peak ($r = -0.785^*$, $p<0.05$) and breakdown ($r = -0.780^{**}$, $p<0.01$) viscosities of waxy sorghum flours, which is consistent with previous findings suggesting that harder sorghum varieties possess a more continuous and extensive protein matrix surrounding the starch granules, leading to lower water absorption, less swelling, and decreased pasting viscosities (Buffo, 1998; Shull et al., 1990).

Conclusion

In this study, waxy sorghum varieties with contamination of normal sorghum starch below 5.0% were effectively selected using iodine staining. All waxy sorghum starches exhibited significantly higher pasting peak and final viscosities than waxy maize starch. Microscopic observations of cooked starch pastes provided visual evidence that the higher peak viscosity of waxy sorghum starches was due to the ability of sorghum starch granules to swell more extensively without losing their granular structure. The ratio of peak 1 (short chains) to peak 2 (long chains) was positively correlated with the peak ($r=0.669^*$, $p<0.05$) viscosities of waxy sorghum starches. The cold storage stability of waxy starches was closely related to the structural characteristics of amylopectin. Starches with lower retrogradation tendency contained a higher

proportion of chains with DP 6-11 (r=-0.684*, p<0.05) and a lower proportion of chains with DP 12-24 (r=0.678*, p<0.05).

Waxy sorghum flours have the potential to be directly used as effective thickeners in food applications. The peak, setback and final viscosities of waxy sorghum flours were significantly positively correlated with the peak (r=0.606*, p<0.05), setback (r=0.635*, p<0.05), and final (0.656*, p<0.05) viscosities of their corresponding sorghum starches. The total starch content in waxy sorghum flours was only significantly correlated with the setback (r=0.694*, p<0.05) and final viscosities (r=0.682*, p<0.05). In contrast, grain hardness exhibited a significant negative correlation with the peak (r =-0.785*, p<0.05) and breakdown (r =-0.780**, p<0.01) viscosities. Thus, selecting waxy sorghum varieties with optimal chain length distribution of amylopectin, higher starch content and lower grain hardness could potentially enhance the commercialization of waxy sorghum as an effective thickener in food applications, maximizing the economic benefits for sorghum breeders.

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Table 3.1 List of waxy sorghum samples provided by the USDA Center for Grain and Animal Health Research (Manhattan, KS).

ARS Label	Product Name	Trait	Sample Number	Location	Replication
F_010	G486	Waxy	21-115	Assaria	1.00
F_016	X20206	Waxy	21-103	Assaria	1.00
F_049	X20217	Waxy	21-113	Assaria	1.00
F_004	A_EXP1	Waxy	21-119	Assaria	1.00
F_046	X20216	Waxy	21-116	Assaria	1.00
F_019	X20207	Waxy	21-104	Assaria	1.00
F_025	X20209	Waxy	21-106	Assaria	1.00
F_029	X20210	Waxy	21-124	Assaria	2.00
F_032	X20211	Waxy	21-125	Assaria	2.00
F_037	X20213	Waxy	21-110	Assaria	1.00
F_041	X20214	Waxy	21-128	Assaria	2.00
F_043	X20215	Waxy	21-112	Assaria	1.00
F_073	X20215	Waxy	21-61	Colby	1.00
F_072	X20214	Waxy	21-60	Colby	1.00
F_074	X20217	Waxy	21-62	Colby	1.00
F_068	X20210	Waxy	21-56	Colby	1.00
F_078	A_EXP1	Waxy	21-68	Colby	1.00
F_076	G486	Waxy	21-64	Colby	1.00
F_065	X20207	Waxy	21-53	Colby	1.00
F_064	X20206	Waxy	21-52	Colby	1.00
F_077	X20216	Waxy	21-65	Colby	1.00
F_067	X20209	Waxy	21-55	Colby	1.00
F_071	X20213	Waxy	21-59	Colby	1.00
F_069	X20211	Waxy	21-57	Colby	1.00
F_240	X20213	Waxy	21-42	Sunray	3.00
F_239	X20212	Waxy	21-41	Sunray	3.00
F_237	X20210	Waxy	21-39	Sunray	3.00
F_234	X20207	Waxy	21-36	Sunray	3.00
F_246	X20216	Waxy	21-48	Sunray	3.00
F_245	G486	Waxy	21-47	Sunray	3.00
F_243	X20217	Waxy	21-45	Sunray	3.00
F_242	X20215	Waxy	21-44	Sunray	3.00
F_236	X20209	Waxy	21-38	Sunray	3.00
F_241	X20214	Waxy	21-43	Sunray	3.00
F_233	X20206	Waxy	21-35	Sunray	3.00

Table 3.2 NIR protein and starch content of sorghum flours, average kernel hardness, diameter, and weight of sorghum grains (data were provided by the USDA Center for Grain and Animal Health Research), and their contamination of normal sorghum starch in waxy sorghum starch.

ARS_Label	NIR Protein dwb%	NIR Starch dwb %	Kernel hardness index	Kernel diameter (mm)	Kernel weight (mg)	Normal sorghum contamination, %
F_010	12.64	70.70	84.49	2.52	20.60	2.70
F_016	12.67	70.80	71.03	2.79	33.63	20.50
F_049	13.68	69.60	70.92	2.82	30.00	5.90
F_004	13.54	69.10	80.28	2.68	26.62	4.90
F_046	13.46	70.10	69.29	2.56	25.94	14.50
F_019	12.56	71.10	70.32	2.76	30.09	3.80
F_025	12.19	71.10	81.72	2.55	21.87	3.60
F_029	12.15	71.40	76.78	2.56	22.43	5.10
F_032	12.81	71.00	75.48	2.49	24.58	1.90
F_037	13.55	69.80	65.80	3.01	35.26	2.30
F_041	13.18	69.70	77.75	2.65	26.77	4.20
F_043	12.78	71.90	73.68	2.65	28.09	32.60
F_073	12.07	73.90	83.70	2.46	21.77	7.00
F_072	12.32	74.41	79.39	2.78	31.39	6.80
F_074	11.41	75.47	79.55	2.55	24.35	11.80
F_068	12.61	72.31	77.66	2.55	24.10	10.70
F_078	12.59	73.85	85.06	2.41	24.21	45.90
F_076	11.85	75.61	86.16	2.42	23.48	16.30
F_065	11.87	74.64	84.20	2.31	21.76	4.20
F_064	11.89	74.52	84.08	2.56	25.94	38.60
F_077	12.04	75.12	89.22	2.42	23.90	40.10
F_067	12.01	73.55	87.75	2.55	20.52	3.10
F_071	11.93	75.03	87.15	2.80	30.78	83.70
F_069	13.96	72.40	85.46	2.82	28.45	7.10
F_240	13.41	72.93	75.29	2.89	33.67	40.80
F_239	12.36	73.69	74.42	2.75	30.23	9.30
F_237	13.00	72.35	72.93	2.76	30.55	15.70
F_234	12.86	73.56	75.57	2.60	30.39	9.80
F_246	13.05	72.66	77.50	2.47	25.92	22.30
F_245	13.47	72.40	78.91	2.60	26.03	36.20
F_243	12.76	73.60	70.80	2.70	29.85	9.60
F_242	12.56	73.13	76.36	2.62	27.70	13.00

F_236	12.57	73.22	85.81	2.66	24.86	5.00
F_241	13.56	72.44	68.78	2.96	36.22	13.20
F_233	12.75	74.34	87.35	2.59	27.87	69.30

Table 3.3 Hardness of ten sorghum grains, particle size and composition of waxy sorghum flours and starches.

		Waxy sorghum flour				Isolated waxy sorghum starch			
Sample	Grain Hardness Index	Average Flour Particle Size (μm)	Total starch (%)	Protein (%)	Ash (%)	Recovery (%)	Ash (%)	Protein (%)	Contamination of normal sorghum starch (%)
F-010	84.5	72.71	83.4±0.6	10.76±0.13	0.855±0.035	79.1	0.129	0.13	2.7
F-032	75.5	73.39	84.3±0.5	11.21±0.13	0.971±0.023	80.5	0.115	0.29	1.9
F-037	65.8	72.77	83.3±0.9	11.35±0.17	1.150±0.032	81.1	0.101	0.28	2.3
F-004	80.3	70.23	83.8±0.9	10.69±0.04	0.793±0.020	79.5	0.139	0.33	4.9
F-019	70.3	67.69	84.1±0.2	10.60±0.05	1.081±0.070	80.8	0.107	0.21	3.8
F-025	81.7	66.73	82.8±0.7	10.52±0.24	1.132±0.014	79.6	0.110	0.37	3.6
F-041	77.8	65.60	81.8±0.9	11.69±0.14	1.024±0.012	82.9	0.123	0.25	4.2
F-065	84.2	71.79	83.6±0.0	10.40±0.02	1.050±0.035	80.5	0.098	0.23	4.2
F-067	87.8	70.94	82.4±0.2	10.87±0.12	1.163±0.015	81.3	0.079	0.35	3.1
F-236	85.8	76.76	84.7±0.7	11.32±0.07	1.058±0.061	81.1	0.109	0.26	5.0
Waxy burgundy		82.02	80.9±1.0	11.07±0.02	1.000±0.047	80.4	0.164	0.31	4.1

*All waxy sorghum samples were milled on a Udy mill with a 1.0-mm screen.

*The starch loss in sorghum grains during the decortication process was accounted for in the recovery calculations.

Table 3.4 Chain-length distribution of waxy sorghum and waxy maize starches.

Starch	Unit chains* (%)				
	DP 6-9	DP 6-11	DP 12-24	DP 25-36	DP ⩾37
F-010	5.79 ± 0.01 bc	15.44 ± 0.04 b	61.08 ± 0.30 a	15.55 ± 0.23 d	7.94 ± 0.12 a
F-032	5.45 ± 0.08 c	14.34 ± 0.04 c	61.14 ± 0.51 a	16.39 ± 0.14 cd	8.14 ± 0.33 a
F-037	6.56 ± 0.22 b	15.65 ± 0.07 b	58.97 ± 0.72 c	17.27 ± 0.59 ab	8.10 ± 0.27 a
F-004	5.57 ± 0.07 c	15.12 ± 0.07 b	61.31 ± 0.16 a	16.21 ± 0.31 cd	7.36 ± 0.08 a
F-019	6.50 ± 0.35 b	15.72 ± 0.31 b	59.34 ± 0.05 bc	16.62 ± 0.35 bc	8.31 ± 0.72 a
F-025	6.12 ± 0.30 bc	15.31 ± 0.34 b	59.37 ± 0.21 bc	17.33 ± 0.13 ab	8.09 ± 0.68 a
F-041	6.11 ± 0.04 bc	15.99 ± 0.02 b	61.35 ± 0.14 a	15.56 ± 0.25 d	7.12 ± 0.41 a
F-065	6.06 ± 0.26 bc	15.80 ± 0.30 b	61.04 ± 0.57 a	15.52 ± 0.11 d	7.66 ± 0.18 a
F-067	5.98 ± 0.24 bc	15.75 ± 0.41 b	61.23 ± 0.16 a	15.81 ± 0.19 cd	7.23 ± 0.77 a
F-236	6.23 ± 0.07 bc	15.28 ± 0.07 b	59.51 ± 0.11 b	17.92 ± 0.01 a	7.30 ± 0.01 a
Waxy burgundy sorghum	5.80 ± 0.10 bc	15.49 ± 0.17 b	60.48 ± 0.76 ab	16.28 ± 0.05 cd	7.74 ± 0.66 a
Waxy maize	7.32 ± 0.28 a	16.67 ± 0.29 a	57.08 ± 0.36 d	17.78 ± 0.08 a	8.47 ± 0.04 a

*Mean values of two measurements; values within the same column followed by the same letters are not significantly different ($p < 0.05$).

Table 3.5 Pasting properties of the isolated sorghum starches and waxy maize starches (6%, w/w).

Starch samples	Pasting properties				
	Pasting temperature	Peak viscosity	Breakdown viscosity	Setback viscosity	Final viscosity
	°C	(cP)	(cP)	(cP)	(cP)
F-010	77.9±0.0 cd	1787±16 e	1017±27 d	300±1 cd	1070±12 cd
F-032	78.7±0.0 b	2042±12 a	1089±12 bc	276±2 ef	1229±2 ab
F-037	77.9±0.0 cd	2069±11 a	1239±17 a	379±6 b	1209±0 ab
F-004	77.9±0.0 cd	1647±11 g	953±3 e	234±2 h	929±6 e
F-019	78.4±0.0 bc	1995±1 b	1127±2 b	378±2 b	1246±1 a
F-025	77.8±0.2 de	1736±6 f	988±4 de	284±8 de	1032±11 d
F-041	78.1±0.4 cd	1990±3 bc	1126±4 b	315±1 c	1179±1 b
F-065	77.6±0.0 def	1934±4 d	1061±11 c	372±13 b	1245±1 a
F-067	77.1±0.0 f	1937±16 d	1096±4 bc	248±1 gh	1089±13 c
F-236	77.2±0.0 f	1951±13 cd	1098±3 bc	259±7 fg	1112±4 c
Waxy burgundy sorghum	77.3±0.3 ef	1807±18 e	1071±16 c	210±2 i	945±0 e
Normal sorghum	91.9±0.0 a	599±1 i	159±4 g	463±13 a	903±8 e
Waxy maize	72.6±0.3 g	1316±27 h	634±26 f	117±4 j	800±57 f

*Mean values of two measurements; values followed by the same letters in the same column are not significantly different ($p < 0.05$).

Table 3.6 Pasting properties of the waxy sorghum flours and waxy maize starch (8%, w/w) measured by Rapid Visco-Analyzer.

Samples	Pasting properties				
	Pasting temperature	Peak viscosity	Breakdown viscosity	Setback viscosity	Final viscosity
	°C	(cP)	(cP)	(cP)	(cP)
F-010	78.2±0.4 b	1365±21 de	506±23 d	555±15 cd	1413±13 d
F-032	79.3±0.1 a	1358±5 e	532±2 d	725±13 a	1551±10 b
F-037	78.9±0.2 a	1499±2 b	603±7 b	736±4 a	1631±1 a
F-004	77.5±0.1 b	1306±3 g	513±1 d	556±1 cd	1349±1 f
F-019	79.1±0.0 a	1423±1 c	571±1 c	715±3 a	1567±1 b
F-025	78.9±0.3 a	1261±1 h	425±13 e	651±19 b	1487±5 c
F-041	77.9±0.0 b	1334±9 f	520±9 d	553±6 cd	1367±6 ef
F-065	78.9±0.1 a	1324±6 fg	523±5 d	583±4 c	1384±7 de
F-067	78.1±0.3 b	1259±11 h	421±6 e	515±17 d	1353±0 f
F-236	78.2±0.4 b	1384±2 d	515±13 d	630±25 b	1499±10 c
Waxy burgundy	79.2±0.1 a	1226±4 i	434±1 e	343±0 e	1135±5 h
Waxy maize starch	71.5±0.0 c	2483±2 a	1430±10 a	195±23 f	1248±15 g

*Mean values of two measurements; values followed by the same letters in the same column are not significantly different ($p < 0.05$).

Table 3.7 Gelatinization properties of waxy sorghum starches and maize starches determined by differential scanning calorimetry.

Starch samples	Gelatinization temperatures				Enthalpy
	To (°C)	Tp(°C)	Tc(°C)	Tc-To(°C)	ΔH(J/g)
F-010	74.8±0.1 bc	78.8±0.1 bc	91.7±0.4 ab	16.9±0.4 cd	21.2±0.1 cd
F-032	75.0±0.1 ab	79.0±0.2 ab	91.1±0.4 bc	16.0±0.4 de	22.2±0.2 a
F-037	74.5±0.0 c	78.5±0.1 c	91.1±0.3 bc	16.6±0.3 cde	21.9±0.1 ab
F-004	74.8±0.1 abc	78.8±0.1 bc	90.6±0.2 cd	15.7±0.2 e	21.9±0.2 ab
F-019	74.7±0.1 bc	78.7±0.1 bc	92.0±0.0 a	17.3±0.1 c	21.4±0.3 bc
F-025	74.6±0.1 c	78.6±0.1 bc	91.3±0.3 ab	16.7±0.4 cd	21.8±0.1 ab
F-041	75.2±0.1 a	79.2±0.1 a	91.6±0.2 ab	16.4±0.3 cde	21.7±0.1 bc
F-065	73.6±0.1 d	77.7±0.1 d	89.7±0.1 e	16.1±0.0 de	21.5±0.1 bc
F-067	72.9±0.1 e	77.1±0.1 e	89.9±0.1 de	17.0±0.1 c	21.3±0.2 cd
F-236	73.0±0.1 e	77.4±0.2 de	90.1±0.0 de	17.1±0.1 c	20.9±0.2 d
Waxy burgundy sorghum	72.7±0.1 e	77.6±0.1 d	91.8±0.1 ab	19.1±0.2 b	20.4±0.1 e
Waxy maize	65.7±0.4 f	73.3±0.2 f	89.5±0.6 e	23.9±0.7 a	19.1±0.3 f

*To, Tp, and Tc represent onset, peak, and conclusion gelatinization temperatures, respectively. Tc – To is the range of gelatinization temperature (the difference between Tc and To); ΔH, gelatinization enthalpies.

Table 3.8 Gelatinization properties of waxy sorghum flours and waxy maize starch determined by differential scanning calorimetry.

Flour samples	Gelatinization temperatures				Enthalpy
	To (°C)	Tp(°C)	Tc(°C)	Tc-To(°C)	ΔH(J/g)
F-010	72.6±0.1 cd	80.4±0.4 cd	96.0±0.3 ab	23.4±0.3 b	12.5±0.2 bcde
F-032	73.6±0.1 ab	80.9±0.2 bc	94.8±0.4 c	21.2±0.3 c	12.9±0.2 bc
F-037	73.6±0.4 ab	81.6±0.1 a	96.3±0.5 a	22.7±0.9 b	12.1±0.3 e
F-004	72.4±0.1 cd	79.7±0.1 ef	94.9±0.2 bc	22.5±0.1 b	12.3±0.1 de
F-019	74.1±0.2 a	81.0±0.3 b	95.4±0.4 abc	21.3±0.3 c	12.7±0.4 bcde
F-025	72.9±0.3 c	80.1±0.1 de	95.4±0.2 abc	22.6±0.5 b	12.8±0.1 bcd
F-041	73.4±0.3 b	80.4±0.2 bcd	95.9±0.6 ab	22.5±0.3 b	13.1±0.2 b
F-065	72.2±0.1 d	78.5±0.4 hi	93.5±0.2 d	21.3±0.3 c	12.3±0.2 de
F-067	70.9±0.1 f	78.3±0.2 i	93.4±0.5 d	22.5±0.5 b	12.4±0.2 cde
F-236	71.5±0.2 e	78.9±0.0 gh	94.5±0.3 c	23.1±0.5 b	12.5±0.1 bcde
Waxy burgundy sorghum flour	70.8±0.1 f	79.3±0.2 fg	95.5±0.4 abc	24.7±0.5 a	12.6±0.1 bcde
Waxy maize starch	65.9±0.2 g	73.4±0.3 j	91.3±0.1 e	25.4±0.2 a	18.9±0.2 a

*To, Tp, and Tc represent onset, peak, and conclusion gelatinization temperatures, respectively. Tc-To is the range of gelatinization temperature (the difference between Tc and To); ΔH, gelatinization enthalpies.

*Mean values of two measurements; values followed by the same letters in the same column are not significantly different ($p < 0.05$).

Table 3.9 Retrogradation properties of gelatinized waxy sorghum starches and waxy maize starches stored at 4 °C for 7 days.

Starch samples	Melting temperatures				Enthalpy
	To (°C)	Tp(°C)	Tc(°C)	Tc-To(°C)	ΔH(J/g)
F-010	38.1±0.2 d	53.0±0.3 a	73.1±0.1 ef	35.1±0.3 bc	14.9±0.1 c
F-032	36.1±0.2 e	52.1±0.4 b	72.4±0.2 fg	36.4±0.1 a	16.5±0.2 a
F-037	38.7±0.1 abcd	53.5±0.6 a	74.7±0.2 a	36.1±0.3 ab	14.6±0.1 cd
F-004	38.2±0.2 cd	53.9±0.0 a	74.3±0.0 abc	36.2±0.2 a	14.7±0.1 cd
F-019	38.9±0.3 abc	53.4±0.3 a	73.3±0.2 de	34.4±0.4 cd	14.6±0.1 cd
F-025	39.1±0.2 ab	53.6±0.3 a	73.6±0.2 cde	34.5±0.1 cd	14.3±0.1 cd
F-041	39.3±0.1 a	53.7±0.2 a	73.9±0.1 bcd	34.6±0.0 cd	15.4±0.2 b
F-065	38.5±0.2 bcd	53.5±0.1 a	72.5±0.2 fg	34.0±0.1 d	14.8±0.1 cd
F-067	38.9±0.5 ab	53.5±0.7 a	74.0±0.4 abcd	35.1±0.9 bc	14.5±0.1 cd
F-236	38.8±0.1 abc	53.5±0.2 a	74.7±0.2 ab	35.9±0.4 ab	14.2±0.3 d
Waxy burgundy	38.5±0.2 bcd	53.2±0.3 a	74.4±0.4 abc	35.8±0.3 ab	14.9±0.3 c
Waxy maize	38.8±0.1 abcd	53.2±0.2 a	72.3±0.1 g	33.5±0.0 d	13.6±0.1 e

*To, Tp, and Tc represent onset, peak, and conclusion melting temperatures of retrograded starches, respectively. Tc – To is the range of melting temperature (difference between Tc and To); ΔH, retrogradation enthalpies.

*Mean values of two measurements; values followed by the same letters in the same column are not significantly different ($p < 0.05$).

Table 3.10 Retrogradation properties of gelatinized waxy sorghum flours and waxy maize starch stored at 4 °for 7 days.

Sorghum flour	Melting temperatures				Enthalpy
	To (°C)	Tp(°C)	Tc(°C)	Tc-To(°C)	ΔH(J/g)
F-010	38.2±0.2 abc	54.1±0.0 ab	69.9±0.4 bc	31.7±0.2 cd	10.2±0.1 de
F-032	38.3±0.6 abc	54.2±0.1 a	69.8±0.2 bc	31.5±0.8 cd	10.9±0.0 b
F-037	37.1±0.3 cde	53.5±0.5 abc	69.6±0.3 bc	32.5±0.0 abc	10.4±0.1 bcde
F-004	37.5±0.5 abcde	53.8±0.2 ab	69.4±0.5 bc	31.9±0.6 bcd	10.0±0.1 e
F-019	38.3±0.3 abc	54.1±0.3 ab	69.3±0.7 c	31.0±0.4 cd	10.1±0.0 de
F-025	38.5±0.3 ab	53.5±0.2 abc	69.1±0.3 c	30.7±0.4 d	10.8±0.2 bc
F-041	37.2±0.3 bcde	54.0±0.3 ab	71.0±0.0 a	33.8±0.3 a	10.1±0.1 de
F-065	37.7±0.3 abcd	53.2±0.0 bc	69.3±0.2 c	31.6±0.1 cd	10.6±0.1 bcd
F-067	36.3±0.3 e	52.8±0.3 c	69.5±0.1 bc	33.2±0.2 ab	10.8±0.0 bc
F-236	37.1±0.2 cde	54.0±0.3 ab	70.6±0.0 ab	33.6±0.2 a	10.4±0.1 cde
Waxy burgundy sorghum flour	38.7±0.1 a	53.4±0.3 abc	69.5±0.4 bc	30.8±0.5 d	10.8±0.0 bc
Waxy maize starch	36.4±0.6 de	51.7±0.2 d	70.0±0.0 abc	33.6±0.6 a	12.6±0.0 a

*To, Tp, and Tc represent onset, peak, and conclusion melting temperatures of retrograded starches, respectively. Tc – To is the range of melting temperature (the difference between Tc and To); ΔH, gelatinization enthalpies.

*Mean values of two measurements; values followed by the same letters in the same column are not significantly different ($p < 0.05$).

Table 3.11 Correlations between chain length distribution of amylopectin, compositional characteristics, pasting and retrogradation properties of starches and flours.

Parameters	DP 6-11	DP 12-24	DP 25-36	DP ≥37	Pk1/Pk2	PaT[a]	PKV[a]	BDV[a]	SBV[a]	FV[a]	ΔH[a]	Grain Hardness	Protein (%)	Total starch (%)	PaT[b]	PKV[b]	BDV[b]	SBV[b]	FV[b]	ΔH[b]
Pk1/Pk2	-0.284	0.451	-0.195	-0.611*	1															
PaT[a]	-0.701*	0.705*	-0.463	-0.302	0.446	1														
PKV[a]	-0.453	0.489	-0.303	-0.292	0.669*	0.805**	1													
BDV[a]	-0.432	0.495	-0.299	-0.345	0.708**	0.842**	0.958**	1												
SBV[a]	-0.198	0.307	-0.325	-0.027	0.393	0.736**	0.786**	0.781**	1											
FV[a]	-0.331	0.377	-0.315	-0.09	0.47	0.717**	0.912**	0.803**	0.893**	1										
ΔH[a]	-0.684*	0.678*	-0.534	-0.121	0.158	0.661*	0.583*	0.479	0.321	0.54	1									
Grain Hardness	0.024	0.489	-0.262	-0.59	0.149	-0.670*	-0.478	-0.616	-0.618	-0.476	-0.237	1								
Protein (%)	-0.266	0.285	0.06	-0.496	0.222	0.148	0.042	0.104	-0.39	-0.221	0.357	-0.175	1							
Total starch (%)	-0.435	-0.264	0.449	0.181	0.228	0.246	0.199	-0.008	0.254	0.371	-0.045	-0.025	0.003	1						
PaT[b]	-0.219	-0.44	0.232	0.772**	-0.238	0.281	0.398	0.336	0.312	0.401	0.262	-0.473	-0.503	-0.047	1					
PKV[b]	0.031	-0.492	0.336	0.389	0.259	0.437	0.606*	0.656*	0.716*	0.643*	0.022	-0.758*	0.104	0.565	0.125	1				
BDV[b]	0.003	-0.29	0.143	0.33	0.183	0.571	0.553	0.559	0.721*	0.649*	0.198	-0.780**	0.212	0.517	0.11	0.927**	1			
SBV[b]	-0.283	-0.483	0.451	0.522	-0.058	0.614*	0.498	0.358	0.635*	0.674*	0.127	-0.781**	-0.069	0.694*	0.248	0.753**	0.670*	1		
FV[b]	-0.219	-0.563	0.509	0.518	0.023	0.52	0.529	0.44	0.635*	0.656*	0.034	-0.746*	-0.087	0.682*	0.232	0.809**	0.666*	0.982**	1	
ΔH[b]	-0.342	-0.014	0.124	0.184	0.056	-0.248	0.13	0.012	-0.301	-0.013	0.205	0.244	-0.338	-0.227	0.587	-0.451	-0.543	-0.128	-0.135	1

* Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level.

Pk1/Pk2, PaT[a], PKV[a], BDV[a], SBV[a], FV[a] and ΔH[a] represent the ratio of peak 1 to peak 2, the pasting temperature, peak, breakdown, setback, and final viscosities, as well as the retrogradation enthalpies of waxy starches, respectively, while PaT[b], PKV[b], BDV[b], SBV[b], FV[b] and ΔH[b] represent the corresponding properties of flours.

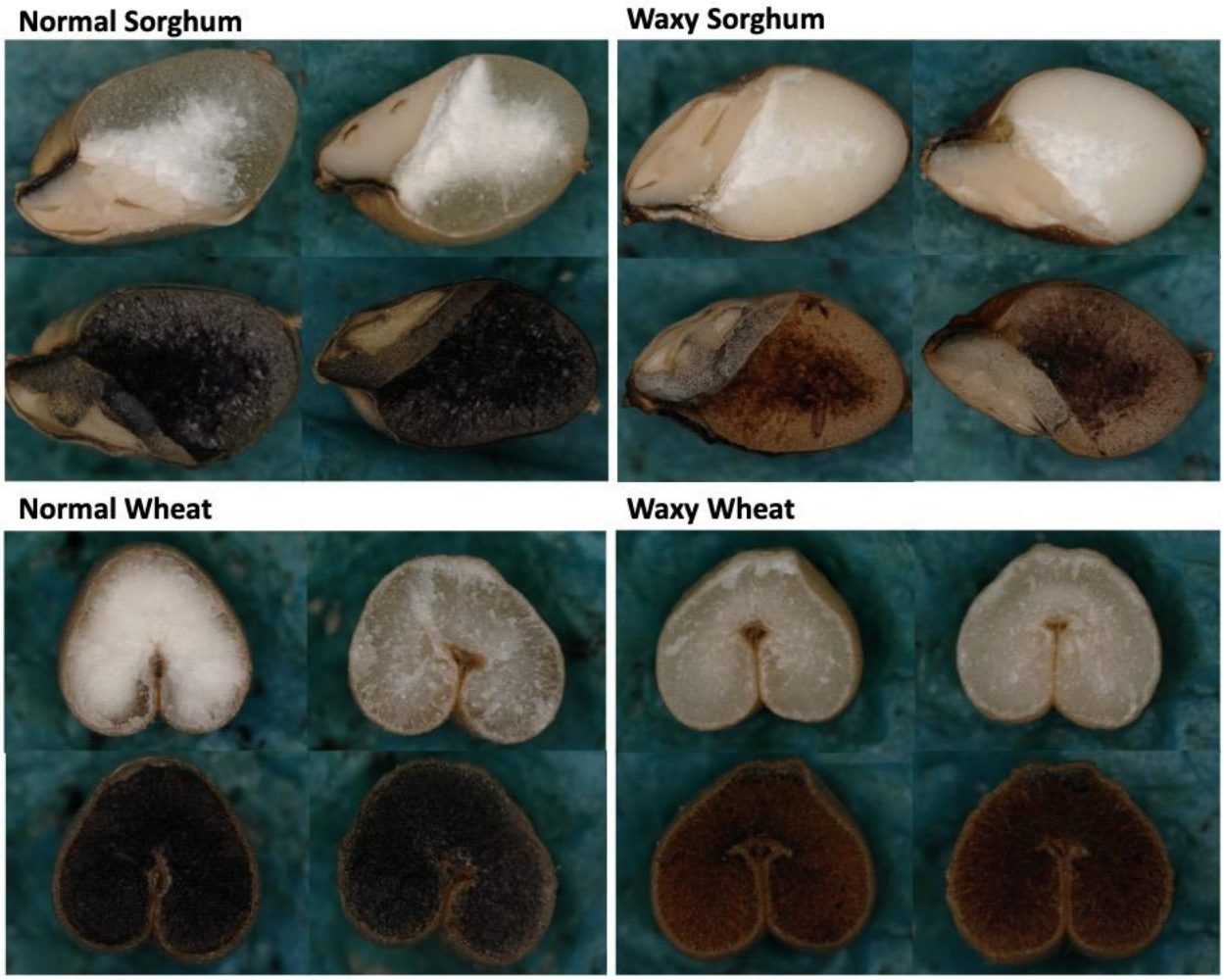

Figure 3.1 Iodine staining of fractured normal sorghum and wheat kernels (stained dark blue) and fractured waxy sorghum and wheat kernels (stained reddish brown).

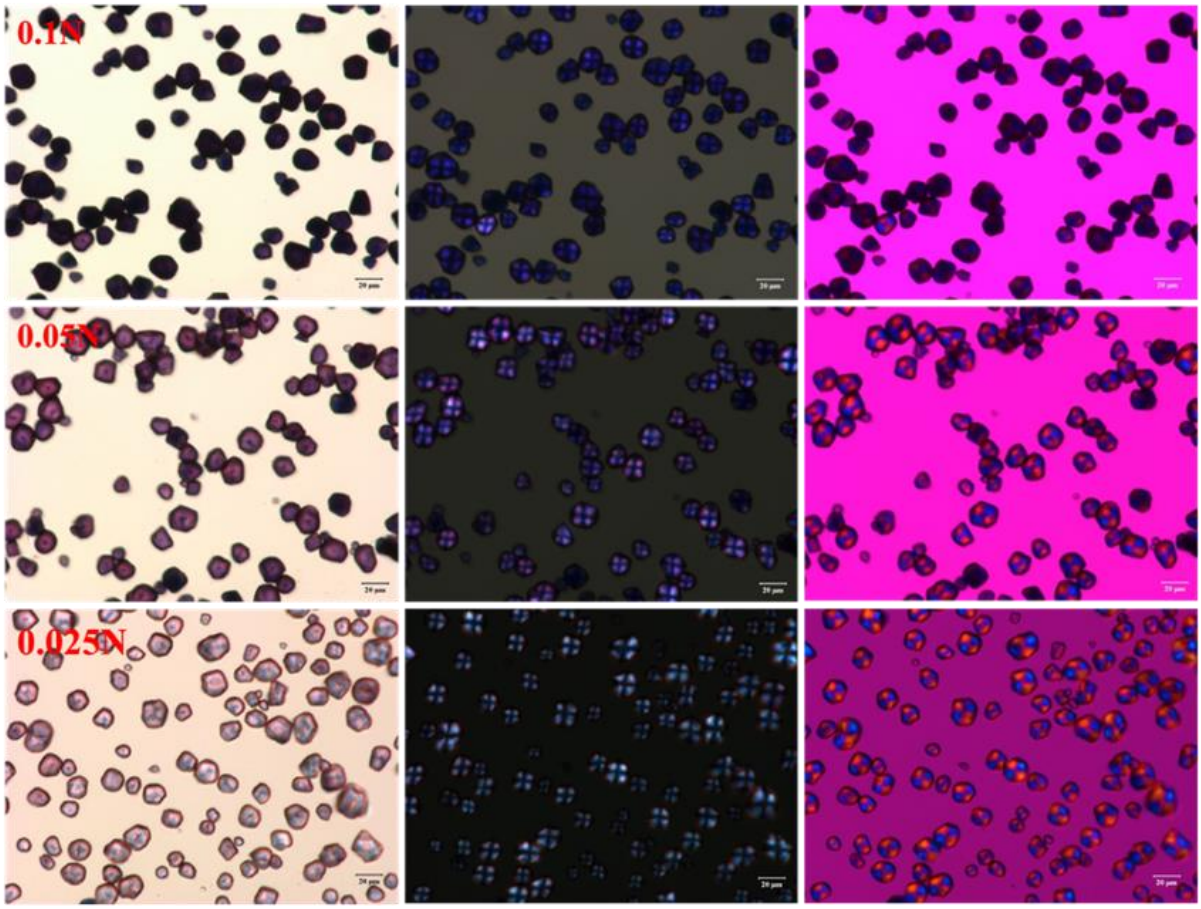

Figure 3.2 Iodine staining of normal sorghum starch (Nulife whole white) at different iodine concentrations (0.1N, 0.05N, 0.025N) under normal light (left), polarized light (middle), and a combination of polarized light and wave plate (right).

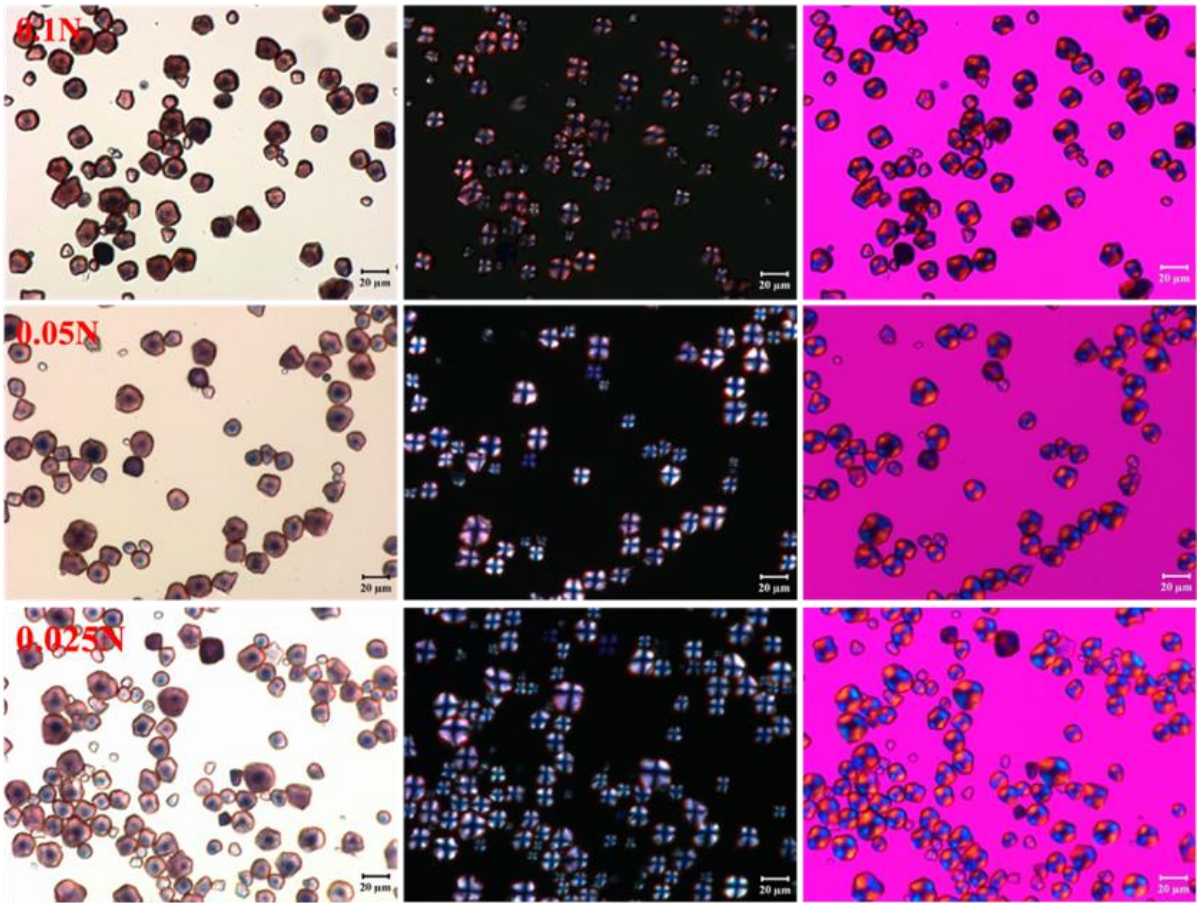

Figure 3.3 Iodine staining of waxy sorghum starch (F-032) with a contamination level of 1.9% at different iodine concentrations (0.1N, 0.05N, 0.025N) under normal light (left), polarized light (middle), and a combination of polarized light and wave plate (right).

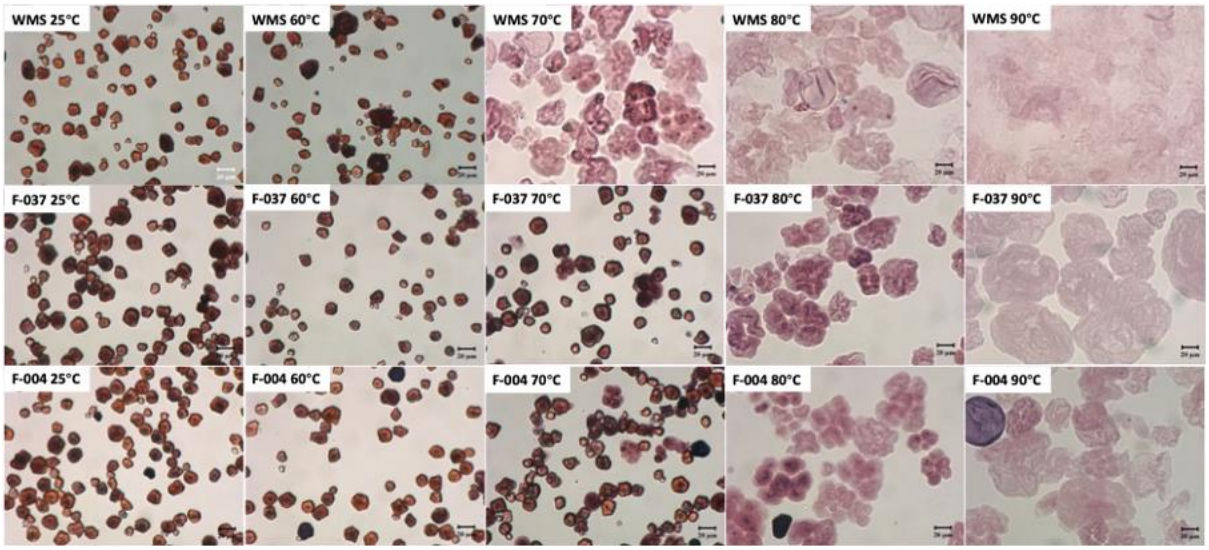

Figure 3.4 Morphology of waxy maize starch (WMS) and waxy sorghum starches (F-037 and F-004), stained with 0.1N iodine solution, and held 20 min at 25 °C, 60 °C, 70 °C, 80 °C and 90 °C.

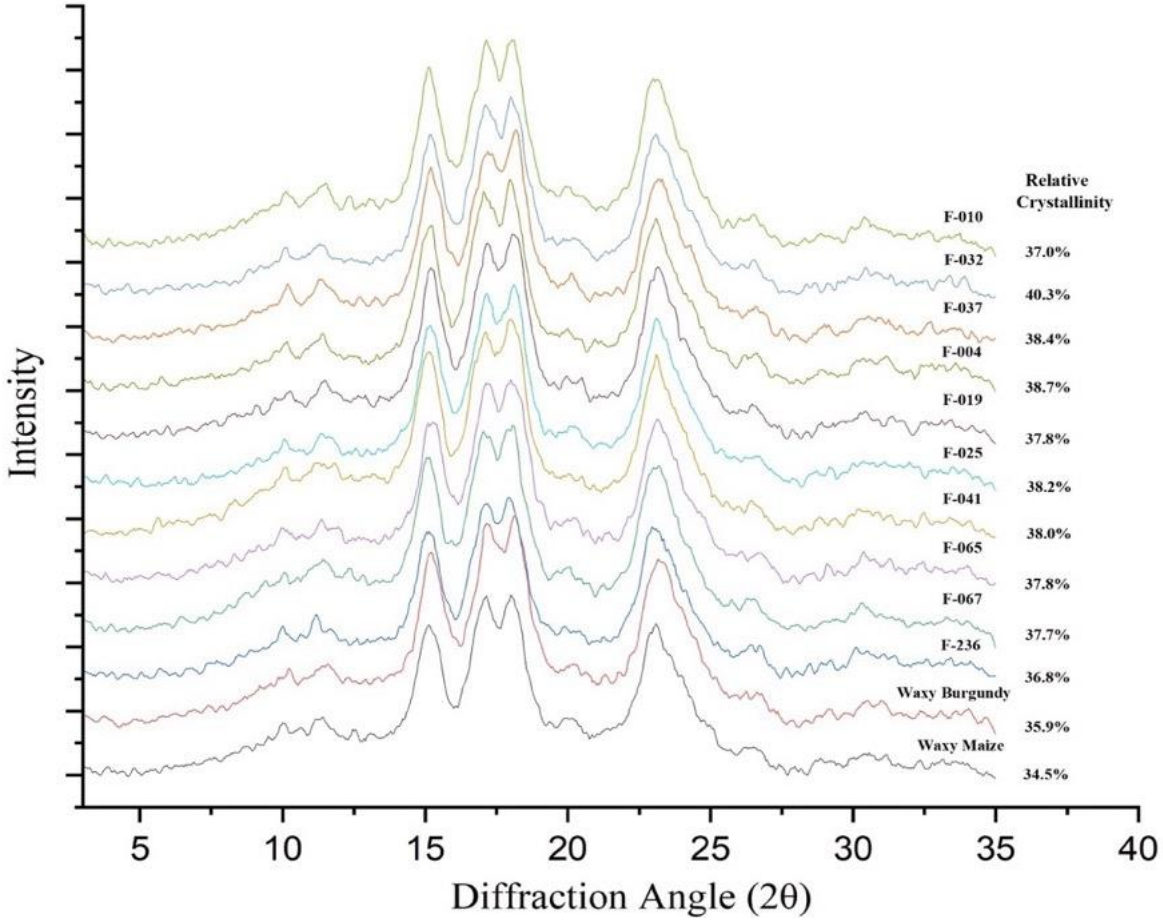

Figure 3.5 X-ray diffraction patterns of waxy starches from sorghum and maize. The relative crystallinity (%) of each sample is given beside the X-ray diffraction curve.

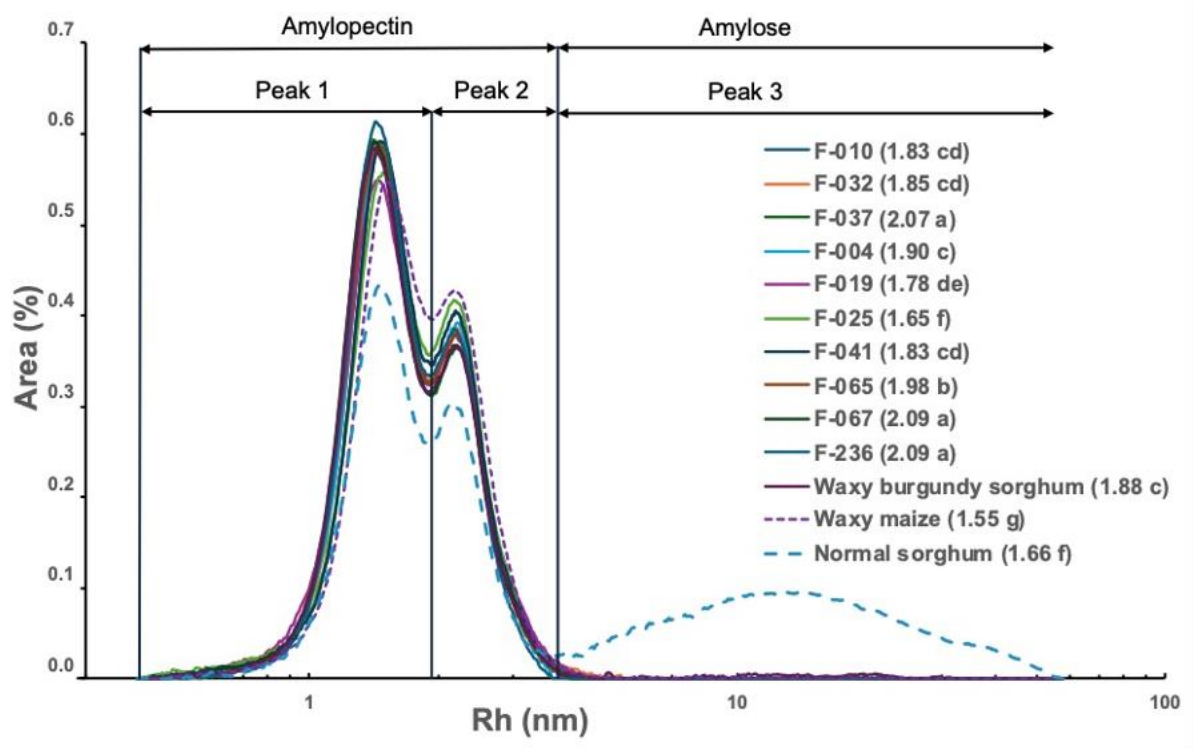

Figure 3.6 Molecular size distribution of the debranched waxy starches from sorghum and maize; Area (%), fractions of debranched starch molecules. Data in the bracket are the ratio of Peak1 to Peak2 ratio.

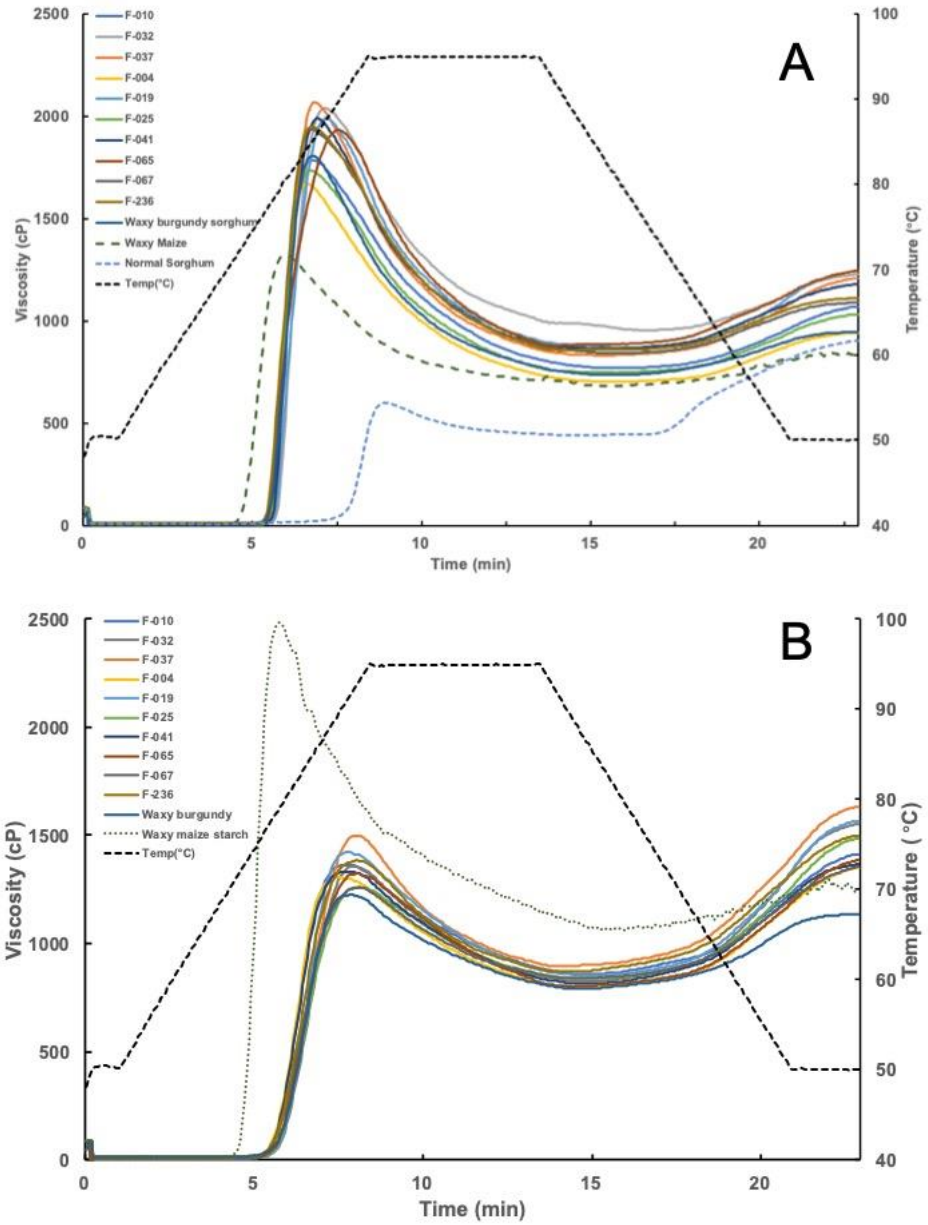

Figure 3.7 Pasting curves of waxy sorghum starches and waxy maize starches (6%, w/w) (A) and waxy sorghum flours and waxy maize starch as reference (8%, w/w) (B) measured by Rapid Visco-Analyzer.

Chapter 4 - Effects of Protein Matrix on Pasting Properties of Waxy Sorghum Flours

Abstract

Waxy sorghum flours, which are non-GMO and gluten-free, would be considered clean-label functional flours and have the potential to be used as food thickeners, especially useful in all-natural foods where chemically modified starches are banned. This study was conducted to investigate the impact of protein matrix on the stability and pasting properties of cooked waxy sorghum flours. The specific objectives were to obtain waxy sorghum flours with different protein levels using enzymatic treatment or solvent extraction methods and to investigate how the protein matrix affects the pasting properties of waxy sorghum flours after cooking by using a confocal laser scanning microscope (CLSM). Results indicated that enzymatic treatment was an effective way to hydrolyze and remove part of the protein and obtain flours with varying protein content compared to the solvent extraction method. Native waxy sorghum flours with higher protein content exhibited lower pasting peak viscosity due to the protein matrix inhibiting starch swelling by forming a physical network around starch granules and restricting the expansion of starch granules during pasting. The hydrolysis of the protein matrix resulted in increased peak pasting viscosities by disrupting the physical network that restricted the swelling of starch granules. These findings show the critical role of the proteins in modulating the functional properties of waxy sorghum flours, providing valuable insights for optimizing their use in food applications, such as effective thickeners and gluten-free products.

Introduction

Sorghum (*Sorghum bicolor* L. Moench) is the fifth most important cereal in total production worldwide, behind maize, rice, wheat, and barley (Bean et al., 2019). It remains a staple food in many parts of the world, particularly in Africa, Asia, and some regions of South America (Ramatoulaye et al., 2016). Research has indicated that its cultivation is economically profitable for both human consumption and industrial applications based on its low cost of production, ability to resist drought, and tolerance for high temperatures (Awika & Rooney, 2004; Boudries et al., 2009). In the United States, sorghum is mainly used for animal feed and ethanol production, with only a small portion used for food applications (Ai et al., 2011; Bean et al., 2019; Sarath et al., 2008). As a gluten-free grain, sorghum serves as a promising alternative flour for individuals with celiac disease and gluten intolerance (Ciacci et al., 2007; Pineli et al., 2015) and has been increasingly utilized in the production of various food products, including fermented beverages, breads, noodles, cookies, tortillas, and porridges (Ciacci et al., 2007; Khoddami et al., 2023; Pineli et al., 2015; Schober et al., 2005; Taylor & Anyango, 2011). Sorghum-based products have been explored extensively due to their unique nutritional properties, including high dietary fiber, antioxidants, and phenolic compounds, which make the grain suitable for developing functional foods and nutraceuticals and contribute to their health benefits (Abah et al., 2020; Awika & Rooney, 2004; Dykes & Rooney, 2006).

The physicochemical and nutritional properties of sorghum flour, which are essential for both food and non-food applications, are influenced by the ratio of amylose and amylopectin within the starch granule, as well as the interactions between starch and non-

starch components, such as proteins and lipids (Bean et al., 2019; Chanapamokkhot & Thongngam, 2007; Khoddami et al., 2023; Sang et al., 2008; Wu et al., 2007). In general, amylose prompts the gelling and film-forming properties of starch and creates highly associated starch. Amylopectin, on the other hand, increases the thickening power of starch and promotes the clarity of starch pastes and gels (Hsieh et al., 2019; Sang et al., 2008). In normal sorghum, most genotypes contain amylose at around 20% to 30% in its endosperm starch, contributing to firmer gel formation and faster starch retrogradation, which can negatively impact the texture and shelf life of some food products (Abah et al., 2020; Zhu, 2014). However, waxy sorghum is essentially composed of amylopectin in its endosperm starch, exhibiting unique pasting and texture properties that could be used as value-added food ingredients and increase its value compared with normal sorghum (Bean et al., 2019; Sang et al., 2008; Zhu, 2014). Mezgebe et al. (2020) demonstrated that waxy sorghum flour has considerable potential to develop gluten-free sourdough fermented flatbread products with good textural functionality due to the slower retrogradation and better water-holding of amylopectin starch. In addition, there has been considerable research into brewing with waxy sorghum (Espinosa-Ramírez et al., 2013; Taylor et al., 2006; Taylor et al., 2013). Pozo-Insfran et al. (2004) suggested that waxy sorghum gelatinizes more rapidly, has a weaker endosperm protein matrix, and is more susceptible to hydrolysis by amylase and protease compared to normal endosperm sorghum, making it more advantageous for brewing.

Waxy sorghum flours have the potential to be used as food thickeners, which would be considered clean-label functional flours and especially useful in all-natural foods where chemically modified starches and flours are banned (De Mesa-Stonestreet et al., 2010;

Hamaker & Bugusu, 2003). Proteins play an important role in the nutritional quality of sorghum for both human consumption and animal feed (Bean et al., 2011). In comparison to other major cereal grains, research on sorghum proteins and their impact on the functionality and quality of sorghum-based foods remains relatively limited. Previous studies have shown that kafirins are located primarily in spherical protein bodies embedded in a glutelin protein matrix and surrounded by starch granules (Choi et al., 2008; De Mesa-Stonestreet et al., 2010; Elkhalifa et al., 2006; Shull et al. 1992; Wu et al., 2007).

Hamaker & Bugusu (2003) employed confocal laser scanning microscopy (CLSM) to visualize the three-dimensional structure of the endosperm protein matrix in native sorghum flour before and after cooking. Their findings revealed that cooking native sorghum flour resulted in the formation of extended web-like or sheet-like protein network, embedding the gelatinized starch granules within. Chanapamokkhot & Thongngam (2007) reported that sorghum flour exhibited lower peak and breakdown viscosities compared to isolated sorghum starch, suggesting that protein-starch interactions restricted the swelling of starch granules and enhanced structural stability. Although waxy sorghum flour has potential as a food thickener, its application may be limited by its grainy texture and unstable paste during storage. In this study, we hypothesized that waxy sorghum flour with a lower protein content could improve textural properties, resulting in a smoother consistency and enhanced thickening efficiency for food applications. Therefore, the objectives of this study were to obtain waxy sorghum flours with different protein levels using enzymatic treatment or solvent extraction methods and to investigate how the protein matrix affects the pasting properties of waxy sorghum flours after cooking by using CLSM.

Materials and methods

Materials

Commercial waxy sorghum grains (waxy burgundy whole grain sorghum) were obtained from NuLife Market (Scott City, KS, USA). The sorghum sample was cleaned by hand to remove foreign materials and then stored at room temperature. Danisco FoodPro alkaline protease, with a specific activity ranging from 580,000 to 650,000 DU/g, was supplied by DuPont Nutrition and Health (Rochester, New York, USA). Waxy burgundy sorghum starch was isolated from decorticated sorghum grains through protein hydrolysis using an alkaline protease based on the method (Huang & Shi, unpublished). All other chemicals were analytical grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Methods

Composition of waxy sorghum flours

Whole sorghum grains were decorticated to remove bran and germ to 20% (by weight), using a tangential abrasive dehulling device (Venables Machine Works, Saskatoon, SK, Canada) equipped with an 80-grit abrasive disk according to Park et al. (2006). Then, the decorticated sorghum grains were milled on a Udy mill with a 1.0-mm screen. The particle size of sorghum flours was determined using a Beckman/Coulter LS 13 320 laser diffraction particle size analyzer (Beckman/Coulter Particle Characterization, Miami, FL) (Xu et al., 2022). The moisture was determined using AACC Approved Method 44-15A, and ash content was determined by AACC Approved Methods 08-01. Protein content (N × 6.25) was obtained by a nitrogen determinator (LECO FP-828, St. Joseph, MI, USA) according to

AACC Method 46–30.01. The total starch content was determined by a Megazyme Total Starch kit (Wicklow, Ireland) by AACC Approved Methods 76-12.

Isolation of waxy sorghum starch

Waxy sorghum starch was isolated using an enzymatic treatment method based on the procedure described by Huang & Shi (unpublished). Briefly, the sorghum grains were decorticated to 20% (by weight) and removed using a tangential abrasive dehulling device (Venables Machine Works, Saskatoon, SK, Canada). The decorticated sorghum grains (100 g, dry basis) were added to 200 mL of water containing 0.32% sodium bisulfite and 0.55% lactic acid and placed in a water bath at 50°C for 48 hours. The steeped kernels were washed with 350ml of distilled water, and the kernels were ground with 150ml of distilled water using a Waring blender (Quaker City, model No. 4E, Straub Co., Warminster, PA) at low speed for 10 mins. The slurry was adjusted to pH 8.0 with 2.0% NaOH solution, and 0.5% alkaline protease was added. During protein hydrolysis, the pH was monitored and kept at a constant level of 8.0 at 45°C for 4h. Subsequently, the pH was adjusted to 4.0 with 1M HCl for 30 mins and then back to 6.0 using 2.0% NaOH. The slurry was centrifuged three times at 3000g for 20min. After that, the sediment (cake) was scraped out and dried in a convection oven at 40 °C overnight.

Preparation of sorghum flours with different protein levels

Enzymatic treatment method

An enzymatic treatment method was used to hydrolyze protein and produce waxy sorghum flours with different protein levels (Figure 4.1). Native waxy sorghum flour (100 g,

dry basis) was mixed with distilled water (~300 ml) in a 500 ml beaker with constant stirring. The slurry was adjusted to pH 8.0 with 2.0% NaOH solution (avoiding gelatinization of starch), and 0.5% FoodPro alkaline protease based on the weight of native sorghum flour was added to hydrolyze protein. During protein hydrolysis, the pH was monitored and kept constantly at 8.0, stirring for 1, 2, or 4 hours at 45°C to produce flours with different protein contents. It was noted that 0.75% alkaline protease was employed for a duration of 4 hours to achieve a higher degree of protein hydrolysis and removal. Subsequently, the pH was decreased to 4.0 by adding 1M HCl for 30 min to deactivate the protease and then readjusted to 6.3 using 2% NaOH, aiming to match the pH level of the slurry made from native waxy sorghum flour and water (1:3). After that, the slurry was centrifuged twice at 4000xg for 15 minutes, with the supernatant carefully removed after each cycle. The sediments (cake) were scraped using a spatula and dried in a convection oven at 40 °C for 24h.

Solvent extraction method

70% ethanol containing 0.5% sodium metabisulfite was used to solublize protein and produce waxy sorghum flours with low protein levels based on the method of Park et al. (2006) with some modifications. Native waxy sorghum flour (100 g, dry basis) was mixed with 70% ethanol (~350 ml) containing 0.5% sodium metabisulfite in a 500 ml beaker with constant stirring for 1 hour at 50°C. The beaker was covered with aluminum foil during the whole process to prevent the evaporation of ethanol. After that, the slurry was cooled down to room temperature and centrifuged at 4000xg for 10 minutes. The sediment was washed with distilled water (400 ml) for 15 minutes and centrifuged at 4000xg for 10 minutes. The whole

washing process was repeated twice. The sediments (cake) were scraped using a spatula and dried in a convection oven at 40°C for 24h.

Pasting properties of waxy sorghum flours

The pasting properties of waxy sorghum flours were determined by a Rapid Visco Analyzer (RVA-4500, Perten Instruments, Sweden). An 8% waxy sorghum flour suspension was prepared by mixing the flour sample 2.24g (dry basis) with 25.76g (corrected by sample moisture) of distilled water. In addition, to study the effect of the protein matrix on the stability and texture of cooked sorghum flour, the pasting properties of waxy sorghum flour with different protein levels treated by alkaline protease were also compared by keeping the same amount of starch and distilled water but with different total solid content. Measurements were performed by following the STD2 temperature profile (Approved Method 76-21.02, Cereals & Grains Association, 2009), where the sample was held at 50 °C for 1 min, heated to 95 °C for 7.5 min, held at 95 °C for 5 min, cooled to 50 °C for 7.5 min, and held at 50 °C for 2 min. The rotating speed of the paddle was 160 rpm. The values of pasting temperature, peak viscosity, breakdown viscosity, setback viscosity, and final viscosity were recorded.

Confocal laser scanning microscopy

To elucidate the changes in the protein matrix of waxy sorghum flours with different protein levels before and after cooking, waxy sorghum flours and the pastes were stained with a protein-specific fluorescent probe, 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde (CBQCA) (Life Technologies, Eugene, OR, USA). The stained sorghum flours and pastes

were examined using CLSM based on the method of Hsieh et al. (2020) with some modifications. For protein staining, uncooked waxy sorghum flours (15mg) or cooked sorghum flours (0.2ml) were suspended in 1.5 ml of 0.1M sodium borate buffer (pH 9.3), after which 15 μL of 20mM potassium cyanide (KCN) and 15 μL of 10 mM CBQCA were added to the suspension in 2ml microcentrifuge tubes. The protein-staining reaction proceeded in the dark for three hours at room temperature. CBQCA-labeled uncooked samples were collected by centrifugation (2000xg, 20min) and washed three times with deionized water (3×1mL). To ensure the protein structure in the cooked samples could be accurately observed, the stained sorghum paste was directly observed under CLSM without undergoing additional washing, which could potentially disrupt the original structure. The stained flour samples were suspended in 0.5ml of distilled water and then observed using a Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss Ltd., Jena, Germany) coupled to an EC Plan-Neofluar 40 × 1.39 oil objective lens on which one drop of immersion oil was applied. The excitation wavelength for the CBQCA channels was set at 488 nm. The three-dimensional micrographs were produced by overlapping more than 20 laser-generated optical planes using Zeiss LSM Image Browser software (Carl Zeiss, Thornwood, NY, USA).

Statistical analysis

Experiments on waxy sorghum flour samples were done in duplicate. Collected data were analyzed using IBM SPSS Statistics Version 26 (IBM Corporation, New York, USA). Analysis of variance (ANOVA) was applied to determine whether data differed significantly from each other ($p < 0.05$). The results were expressed as mean and standard deviations (SD).

Results and Discussion

Preparation of waxy sorghum flours with different protein levels

Protease enzymes are commonly used in the isolation of starch from cereal grains to achieve higher-purity starch with minimal residual protein (Chew-Guevara et al., 2016; Dorglamud et al., 2013; Lumdubwong & Seib, 2000; Sittipod & Shi, 2016; Tester et al., 2007). Despite the strong interaction between starch and the protein matrix in sorghum, protease treatment can still effectively hydrolyze proteins, facilitating the release of starch granules (Chew-Guevara et al., 2016; De Mesa-Stonestreet et al., 2010; Mezo-Villanueva & Serna-Saldívar, 2004). In this study, alkaline protease was utilized to hydrolyze protein in sorghum flours, resulting in a significant decrease in protein content compared to the unmodified waxy sorghum flour (Figure 4.3). The same protease has been successfully applied to hydrolyze proteins in wheat flour, facilitating the extraction of non-starch lipids and demonstrating its effectiveness in disrupting the protein matrix in different cereal flours (Abdul Manan et al., 2022). It should be noted that the efficiency and extent of protein hydrolysis could be influenced by various factors, such as protease type and specificity, enzyme concentration, reaction conditions (pH, temperature, and time), and substrate properties (Tavano, 2013). Protein hydrolysis using 0.1% protease for 1 to 2 hours, with a specific activity ranging from 580,000 to 650,000 DU/g, did not significantly reduce the protein content in the recovered sorghum flours likely due to the low enzyme concentration. In contrast, applying 0.5% or 0.75% protease for protein hydrolysis in waxy sorghum flour resulted in a significant reduction in the protein residues of the recovered flours (Figure 4.3). The results indicated the effectiveness of alkaline protease in breaking down protein into

smaller peptides and amino acids, which were subsequently removed during washing steps (Figure 4.1). Waxy sorghum flours with different protein levels were used to investigate the impact of the protein matrix on the pasting and stability of cooked waxy sorghum flours. The ability to adjust protein levels provides an effective approach to optimizing the functional properties of waxy sorghum flour for specific food applications.

Pasting properties of waxy sorghum flour and isolated starch

The pasting profiles of both unmodified waxy sorghum flour and isolated starch are shown in Figure 4.4 under two conditions: (A) with a constant total solid content (8%) but different starch content and (B) with the constant starch content but different solid contents in total. In general, waxy starches from cereal grains are characterized by higher peak viscosities than amylose-containing starches, followed by a more pronounced breakdown and a lower degree of setback after pasting (Abdel-Aal et al., 2002; Hung et al., 2007; Zeng et al., 1997). Peak viscosity is related to the degree of swelling of starch granules during heating, while breakdown indicates the stability of starch granules under high shear conditions (Ragaee & Abdel-Aal, 2006). The pasting properties of waxy sorghum flours could be affected by several factors, such as starch concentration and composition, and the presence of non-starch components, such as protein and lipid (Batey & Curtin, 2000; Bean et al., 2011; Ragaee & Abdel-Aal, 2006; Zhang & Hamaker, 2005). In this study, isolated waxy sorghum starch exhibited significantly higher pasting viscosities than unmodified waxy sorghum flour under both experimental conditions. At a total solid content of 8%, waxy sorghum starch exhibited significantly higher pasting viscosities due to its higher starch content, resulting in more granules to absorb water and swell (Figure 4.4A). On the other hand, when the starch

content remained constant, waxy sorghum starch still exhibited higher pasting viscosities than waxy sorghum flour, despite the flour having higher total solid content (Figure 4.4B). Chanapamokkhot & Thongngam (2007) also found that normal sorghum flour had significantly lower peak and breakdown viscosities compared to the isolated sorghum starch. These results both suggest that the presence of non-starch components, especially protein matrix in sorghum flour may inhibit the swelling and gelatinization of starch granules and enhance their resistance to shear-induced breakdown (Chandrashekar & Kirleis, 1988; Hamaker & Bugusu, 2003). Further research was conducted to investigate the impact of protein matrix on the pasting properties of waxy sorghum flours.

Effect of protein matrix on the pasting properties of waxy sorghum flours

The pasting properties of waxy sorghum flours with different protein levels are shown in Figure 4.5 under two conditions: (A) with the same total solid content (8%) but different starch content and (B) with the same starch content but different total solid contents. Sorghum proteins, primarily kafirins, are encapsulated within tightly packed protein bodies embedded in a glutelin protein matrix, which is further surrounded by starch granules (De Mesa-Stonestreet et al., 2010). In this study, protease treatment was demonstrated to effectively hydrolyze sorghum protein and had a significant impact on the pasting properties of waxy sorghum flour. Waxy sorghum flours with reduced protein content exhibited significantly higher pasting viscosities compared to the unmodified waxy sorghum flour under both experimental conditions. When the total solid content was maintained at 8%, waxy sorghum flours with lower protein levels exhibited significantly higher pasting viscosities due to its higher starch content, leading to water absorption and greater viscosity

development (Figure 4.5A). Unmodified waxy sorghum flour had the lowest pasting viscosities due to the lowest starch and highest protein content.

In contrast, with same starch content but variations in total solid content, unmodified waxy sorghum flour still exhibited significantly lower pasting peak viscosities than waxy sorghum flours with lower protein content (Figure 4.5B). The results suggest that the reduced protein could possibly facilitate this process by allowing starch to absorb more water during pasting, minimizing physical barriers around starch granules, and reducing starch-protein interactions. In our study, the CLSM micrographs show that the protein matrix in native waxy sorghum flour appeared to develop into extended, web-like structures with starch granules embedded within the network after cooking (Figure 4.7). The protein matrix could compete for water, form barriers around starch granules, and interact with starch molecules, thereby inhibiting starch swelling (Hamaker & Bugusu, 2003). The enzyme-treated flours with reduced protein content exhibited higher breakdown viscosity, suggesting they were more susceptible to shear due to the absence of a protective protein matrix. The findings highlight the critical role of the protein matrix in modulating starch pasting behavior in waxy sorghum flour.

Pasting properties of waxy sorghum flours with low protein (solvent extraction)

When 70% ethanol and a reducing agent (sodium metabisulfite) were used to extract sorghum protein, waxy sorghum flour with 4.46% protein exhibited a higher peak viscosity compared to waxy sorghum flours with 5.37% or 2.83% protein extracted using enzymatic treatment (Figure 4.6). This could be explained by the fact that the reducing agent broke disulfide bonds within protein molecules, facilitating more extensive solubilization of protein in the

70% ethanol and reducing the protein-starch interaction and allowing greater swelling and resulting in higher peak viscosities. In contrast, enzymatic treatments may leave residual protein or protein fragments that interfere with starch swelling, resulting in lower viscosities.

Confocal laser scanning microscopy of protein in waxy sorghum flour

To investigate the interactions between sorghum proteins and starch granules in uncooked waxy sorghum flours and to examine how sorghum proteins impact functionality in cooked pastes, CLSM was used to visualize protein structures. CLSM is a form of light microscopy in which white light or a narrow range of wavelengths of laser light excites a specific fluorescence material to investigate the microstructural changes in foods (Choi et al., 2008). The protein dye, 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde (CBQCA), fluoresces only after reaction with primary amines in proteins via covalent bonds, which makes it particularly useful in this application. 3D images comprising more than 20 laser-generated optical planes of stained protein in unmodified waxy sorghum flours and the isolated waxy sorghum starch before and after cooking are shown in Figure 4.7.

Protein in uncooked sorghum flours is shown in green surrounding dark areas where starch granules exist (Figure 4.7A). Protein bodies were seen as small circular bodies embedded in a larger glutelin protein matrix, most likely due to the incomplete penetration of the CBQCA dye, thus highlighting their spherical structure. The protein bodies are 0.4 to 2μm in diameter, with an outer shell composed mainly of crosslinked β- and γ-kafirins and an interior comprised predominantly of α-kafirin (Duodu et al., 2003). After water-washing, albumins and globulins (water-soluble proteins) could be dissolved in water and removed during centrifuge. The removal of water-soluble proteins reduced the fluorescence intensity

(Figure 4.7B) and contributed to higher pasting peak viscosity compared to native sorghum flour. After cooking, the proteins in unmodified and water-washed sorghum flours appeared to develop into extended, web-like, or sheet-like structures, with starch granules embedded within the network (Figure 4.7G &H). The development of extensive web-like protein networks during the cooking of waxy sorghum flour paste seems to be associated with disulfide-mediated polymerization (Hamaker & Bugusu, 2003). These results show that proteins played a significant role in developing extended structures, such as web-like and sheet-like structures primarily composed of this protein group.

Enzymatic treatment of waxy sorghum flour resulted in the partial hydrolysis of proteins, reducing their association with starch granules and modifying the functional properties of the flour. Protease treatment for 1 or 2 hours partially disrupted the protein-starch association, as the relatively short treatment duration did not fully hydrolyze all proteins, leaving residual intact proteins or protein fragments adhering to the starch granules and maintaining a partial association (Figure 4.7C&D). The breakdown of the protein layer around starch granules partially exposed the starch surface, making it more accessible to water, resulting in higher peak viscosities compared to native or water-washed sorghum flours. Interestingly, the remaining protein may still interact to form a weaker network, providing some level of inhibition to starch swelling, though to a lesser extent than that in untreated flours (Figure 4.7I&J). Compared to protein hydrolysis for 1 or 2 hours, waxy sorghum flours treated with 0.5% or 0.75% concentration of protease for 4 hours resulted in more extensive protein hydrolysis, significantly reducing the amount of residual protein

associated with starch granules (Figure 4.7E&F). As a result, the flours exhibited increased starch swelling and gelatinization during cooking due to the absence of protein barriers.

Conclusion

The application of alkaline protease treatment effectively hydrolyzed the protein in waxy sorghum flour, and the sorghum flours with lower protein exhibited higher pasting viscosity and less grainy texture. CLSM results show that the protein matrix in native sorghum flour could inhibit starch swelling by forming a protein network (web-like or sheet-like structures) around starch granules and restricting the expansion of starch granules during cooking. Waxy sorghum flours with more extensive protein hydrolysis exhibited increased starch swelling and gelatinization during cooking due to the absence of protein barriers. This study demonstrates the potential to tailor the functional properties of waxy sorghum flour for specific industrial applications requiring higher pasting viscosities, such as sauces, soups, and bakery fillings, offering insights into the interaction between proteins and starch in complex food systems. Furthermore, it provides a valuable understanding of the complex interactions between protein and starch in food systems.

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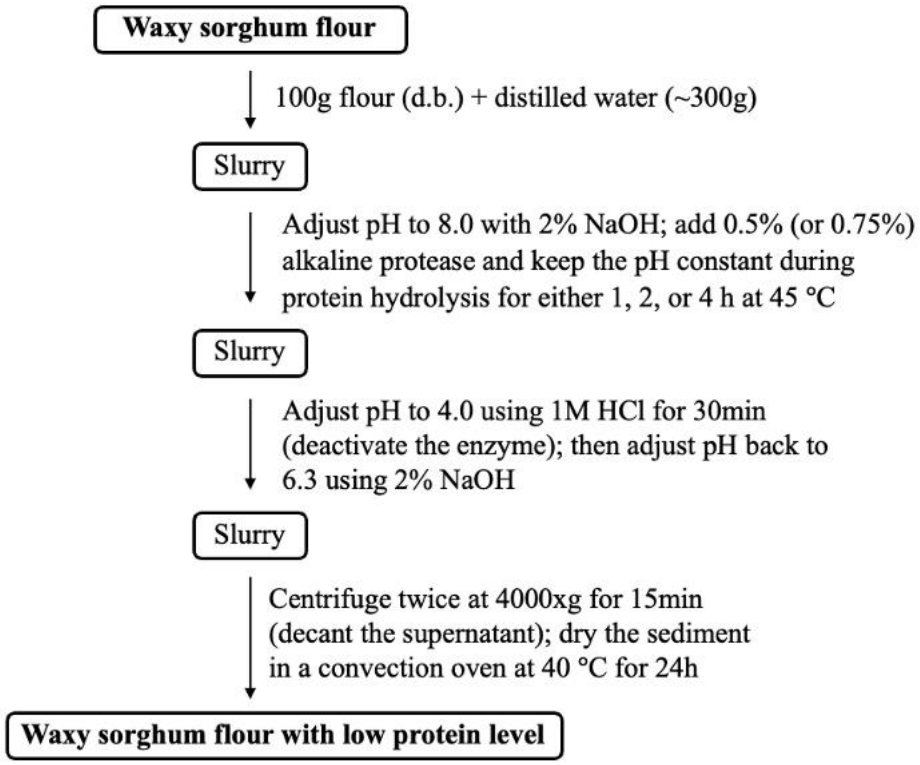

Figure 4.1 The enzymatic treatment procedure using alkaline protease to prepare waxy sorghum flours with different protein levels.

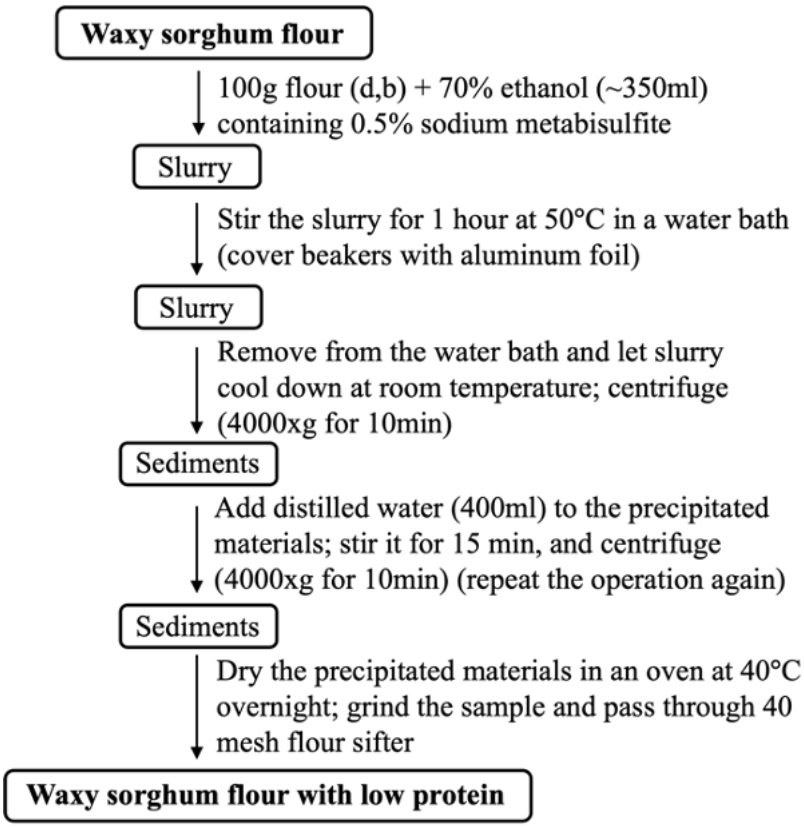

Figure 4.2 The solvent extraction procedure using 70% ethanol plus 0.5% sodium metabisulfite to prepare waxy sorghum flours with different protein levels.

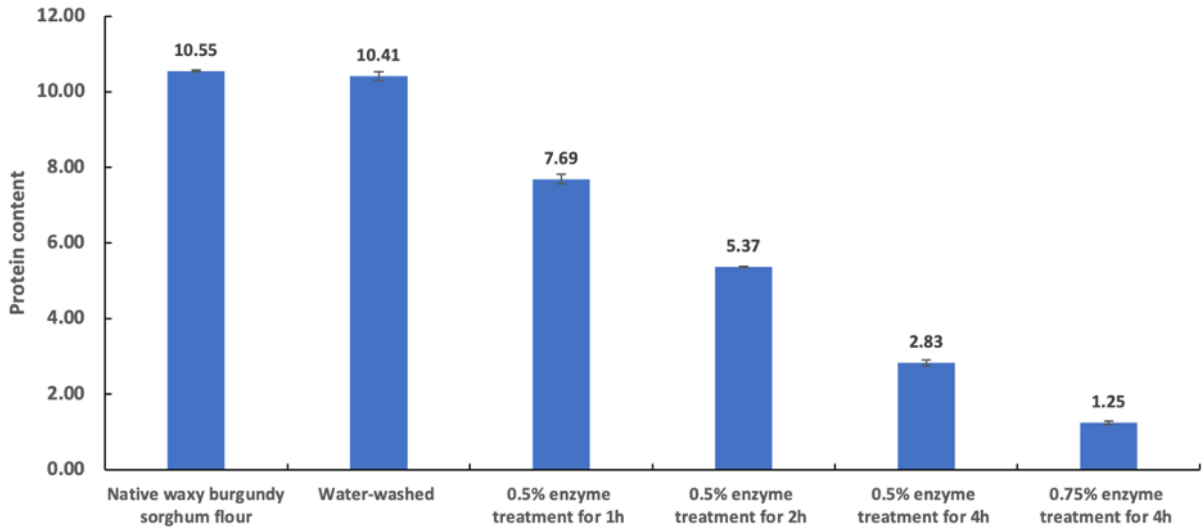

Figure 4.3 The protein content of native waxy sorghum flour, water-washed, and enzyme-treated waxy sorghum flours (0.5% for 1h, 2h, and 4h; 0.75% for 4h).

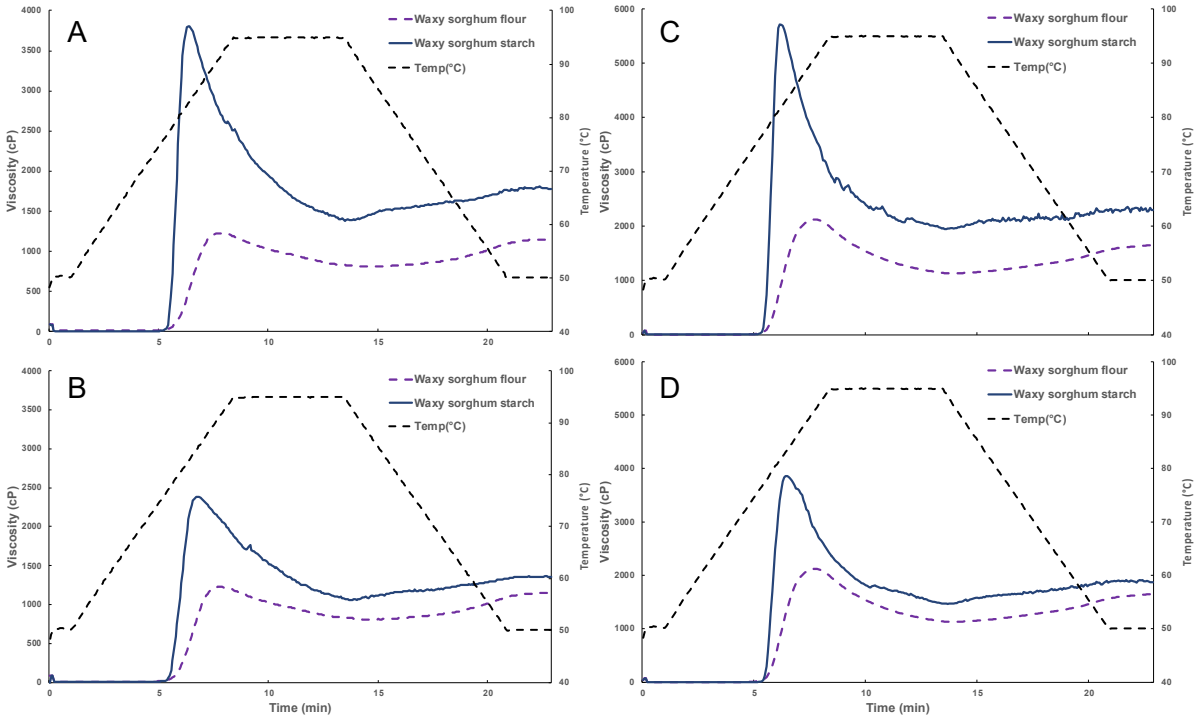

Figure 4.4 Pasting curves of unmodified waxy burgundy sorghum flour and the isolated waxy sorghum starch measured by Rapid Visco-Analyzer (A: Total solid content maintained at 8%, with differing starch content, B: Starch content kept constant, with the difference in total solid content; C: Total solid content maintained at 10%, with differing starch content, D: Starch content kept constant, with the difference in total solid content).

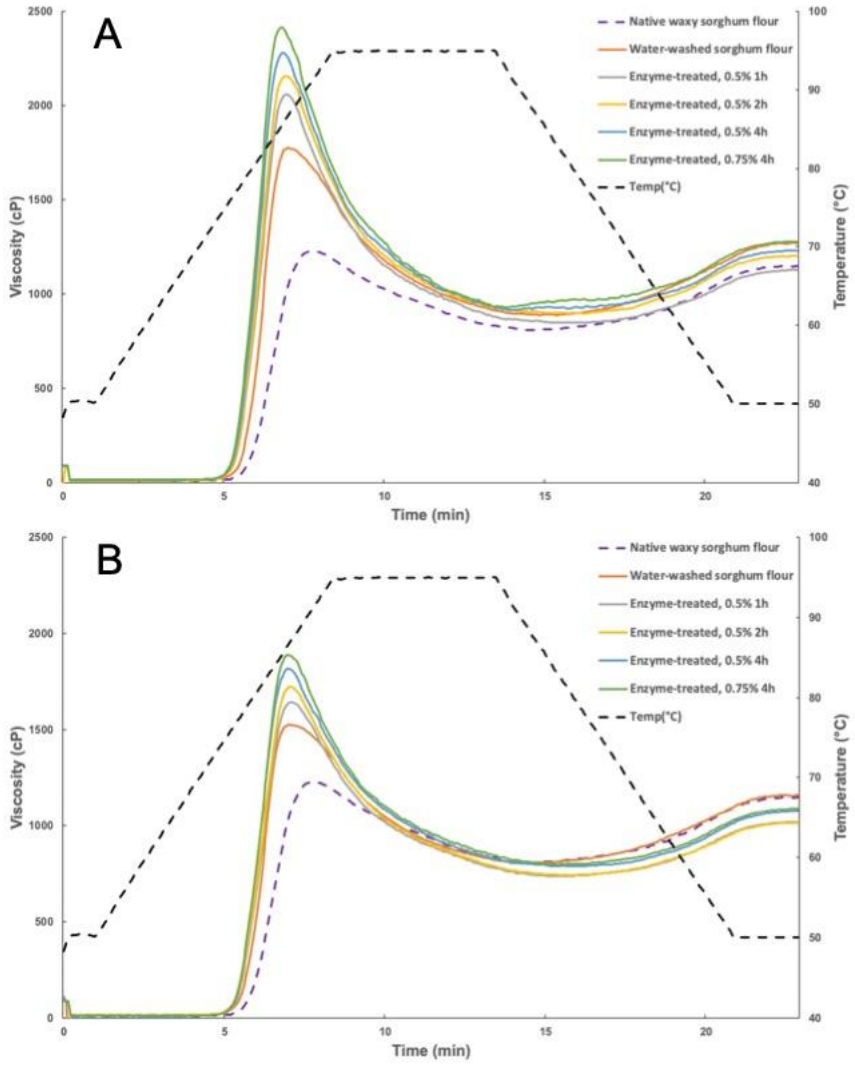

Figure 4.5 Pasting curves of waxy burgundy sorghum flour with different protein levels. (A: Total solid content maintained at 8%, with differing starch content; B: Starch content kept constant, with the difference in total solid content).

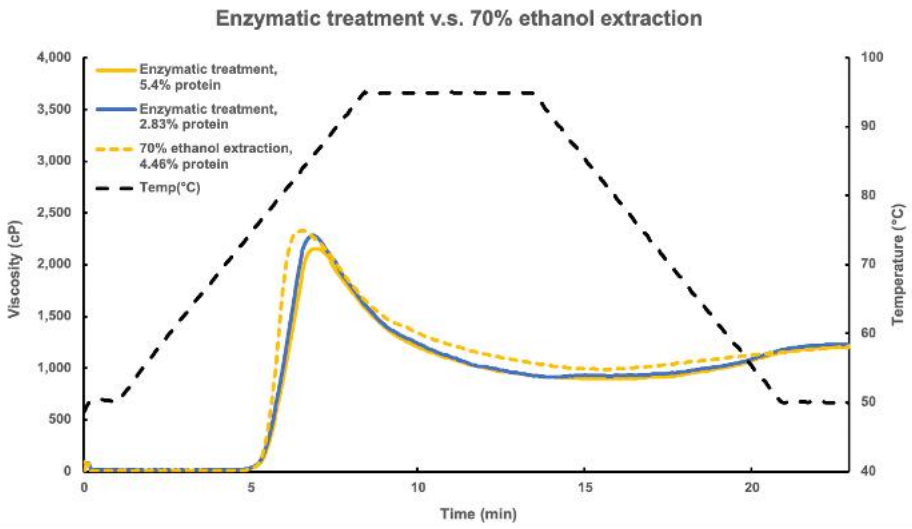

Figure 4.6 The pasting curves of waxy burgundy sorghum flours with 5.4% and 2.83% protein by enzymatic treatment and with 4.46% protein by 70% ethanol extraction (8%, w/w).

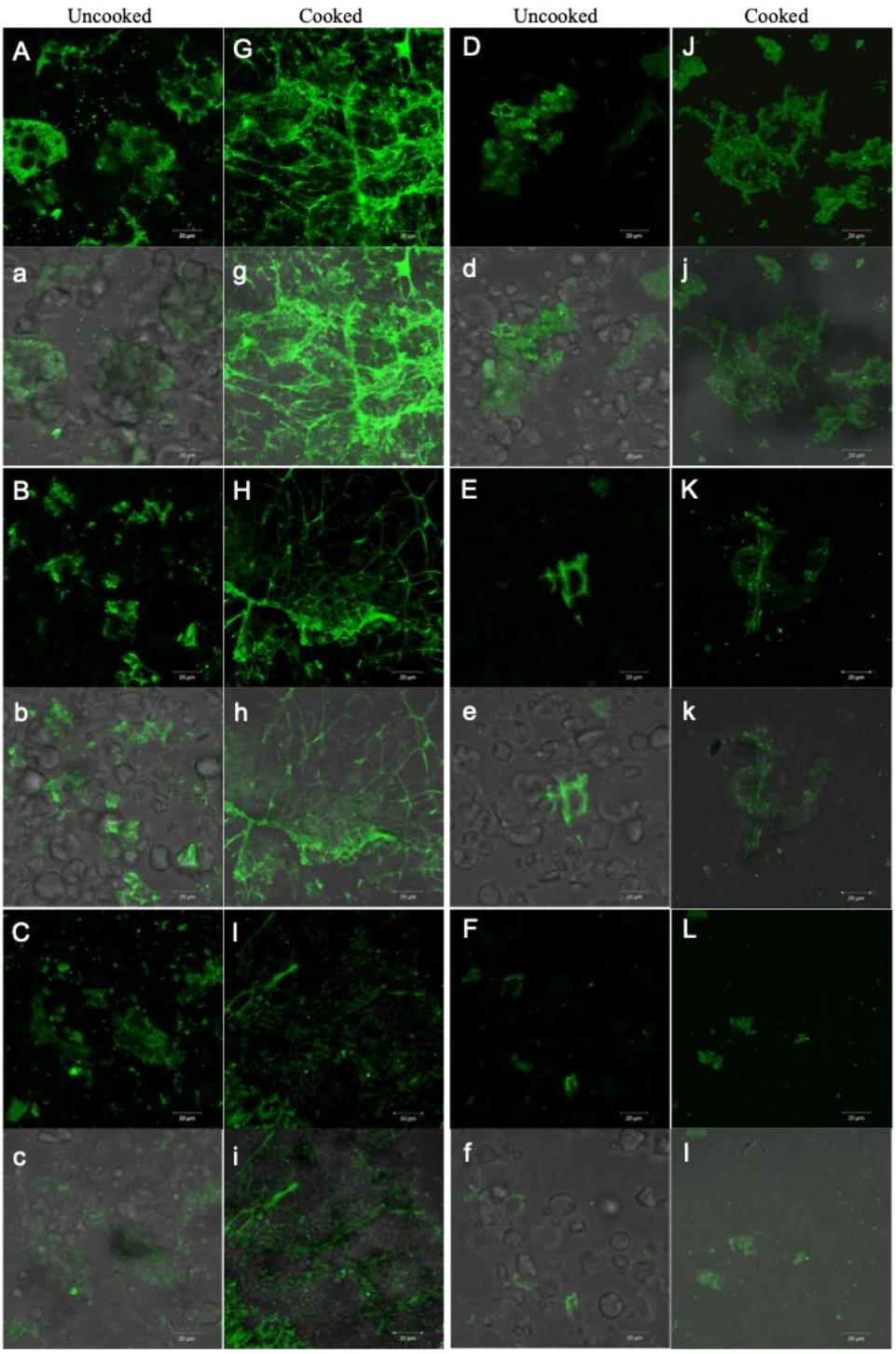

Figure 4.7 Confocal microscopic images of the uncooked (A-F) and cooked (G-L) waxy sorghum flours with different protein levels (A: Native sorghum flour with 10.55% protein; B: Water-washed flour with 10.41% protein; C: Enzyme-treated sorghum flour with 7.69% protein; D: Enzyme-treated sorghum flour with 5.37% protein; E: Enzyme-treated sorghum flour with 2.83% protein; F: Enzyme-treated sorghum flour with 1.25% protein).

Chapter 5 - Conclusions and Perspectives

Enzymatic treatment of decorticated sorghum could be used as an effective and efficient method for starch isolation, producing starch with higher purity, improved color quality, and higher pasting viscosities compared to traditional wet milling. Waxy sorghum varieties with contamination of normal sorghum starch less than 5.0% were effectively identified using iodine staining, and all isolated waxy sorghum starches exhibited significantly higher peak and final viscosities than waxy maize starch. The cold storage stability of waxy sorghum starches was closely related to the structural characteristics of amylopectin. Starches with lower retrogradation tendency contained a higher proportion of chains with DP 6-11 and a lower proportion of chains with DP 12-24.

Waxy sorghum flours also show the potential to be directly used as thickeners in food applications. Selecting waxy sorghum varieties with optimal chain length distribution of amylopectin, higher starch content and lower grain hardness could potentially enhance the commercialization of waxy sorghum as food thickeners, maximizing the economic benefits for sorghum breeders. In the last study, CLSM results show that the protein matrix in unmodified waxy sorghum flour could inhibit starch swelling by forming a physical network around starch granules and restricting the expansion during cooking. Alkaline protease treatment effectively hydrolyzed the protein, reducing the physical barriers to starch swelling and resulting in higher viscosity and smoother texture. This research provides a valuable understanding of the complex interactions between protein and starch of sorghum in food systems.

Future research could focus on selective breeding strategies to develop waxy sorghum lines with a higher proportion of short amylopectin chains (DP 6-11) and a lower proportion of chains (DP 12-24) to enhance the cold storage stability of sorghum-based food products. Identifying and manipulating key genes involved in starch biosynthesis, such as starch synthases and starch branching enzymes, may provide better control over amylopectin structure to optimize its functional properties. Additionally, future studies should examine a diverse range of waxy sorghum genotypes to gain a deeper understanding of the relationship between starch structure and functional properties. Investigating the correlations between pasting properties, molecular size distribution, and internal and external chains of amylopectin may provide valuable insights for improving the industrial applications of waxy sorghum.