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Nanostructure of Sorghum Starch Granules as Studied by Small Angle X-Ray Scattering (SAXS)

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Abstract

Sorghum, one of the staple crops in arid regions of the world, has been shown to be less digestible than other sources of starch. Physiochemical properties of starch, such as size of granule and crystallinity can affect the digestibility of the starch (Singh et al., 2010a). This experiment aimed to describe these physiochemical properties of red and white sorghum starch as they compare to potato starch using light microscopy and Small Angle X-Ray Scattering (SAXS). The light micrographs of the starches showed no significant difference ($p>0.05$) in diameter size among potato, red sorghum, and white sorghum starches. The lamellar repeat distance of starch granules is indicated using SAXS by a small peak at $q\sim0.63$. However, it was found that red and white sorghum have a q value of $0.58 \pm 8.9\times10^{-4}$ and $0.57 \pm 1.3\times10^{-3}$ respectively, while potato starch had a q value of $0.62 \pm 2.0\times10^{-3}$. The smaller q values of sorghum translated into a larger d spacing as compared to potato starch. This could imply a larger amount of crystallinity and resistance to gelatinization in sorghum which could be a reason for sorghum's high amount of total resistant starch.

Introduction

Sorghum, one of the staple crops of Africa, Asia, and Latin America, has been shown to be enzymatically less digestible than other grains like rice, wheat, and barley (Dicko et al., 2006; Mukisa et al., 2012). It has been shown that the total amount of resistant starch in potato starch is 10%, while the total amount of RS in normal sorghum starch is 19% (Singh et al., 2010a). Starch is the largest component of sorghum comprising around 69.5 % of the granule on average (FAO, 1995).

This project aims to use light microscopy and Small Angle X-Ray Scattering (SAXS) to help define the microstructure of sorghum starch granules, and thus help understand the physiochemical reasons for its starch indigestibility.

Starch is the main energy storage component in plants, including sorghum (Doutch and Gilbert, 2013). The largest source of carbohydrate in the human diet comes from starch (Singh et al., 2010a; Doutch and Gilbert, 2013). Native starch granules are insoluble and comprised of two main polysaccharides: amylose and amylopectin (Pikus, 2005; Cameron and Donald, 1992; Sanderson et al., 2006). Amylose is a linear glucose polymer of α-D-glucopyarnose units linked α-1,4, and amylopectin is a branched glucose polymer of α-D-glucopyarnose units linked α-1,4, and α-1,6 (Singh et al., 2010b).

Starch granules across different botanical sources can be polymorphic (some polyhedral, some elliptical, some spherical), and polydisperse. However, all starch granules are made of concentric amorphous and semi crystalline 'growth' rings (Jenkins and Donald, 1996). The amorphous and semicrystalline rings have been shown to have a different susceptibility to amylolytic attack (Jenkins et al., 1993). Amylopectin is more susceptible to amylolytic attack because of its larger surface area and branched structure (Singh et al., 2010a).

Cameron and Donald described the structure of the semicrystalline layer to have alternating crystalline and amorphous layers (1992), however the nomenclature was changed in order to more accurately describe the structure (Daniels and Donald, 2003). Daniels and Donald renamed the crystalline and amorphous components to the mesogen and spacer units respectively (2003). This was done to account for the transverse bending of each layer (Daniels and Donald, 2003).

Gelatinization is the process where water is taken up by starch granules in the presence of excess water and heat, or mechanical shear (Cameron and Donald, 1992; Waigh et al., 2000). The process of gelatinization makes starch easier to digest. During gelatinization the birefringence, due to the semi-crystalline nature of the granule, is lost (Jenkins and Donald, 1998). Water is taken up through the amorphous regions of the granule, and the granule eventually swells and bursts (Jenkins and Donald, 1998). The exact time in which gelatinization occurs is difficult to assess as each granule will swell and burst at different rates (Jenkins and Donald, 1998). In the past the end point of gelatinization was determined by the time in which birefringence was lost, or birefringence end point temperature (BEPT) (Jenkins and Donald, 1998). SAXS can help determine the exact point of gelatinization at different temperatures by assessing the loss of crystallinity in the starch granule over time.

It has been shown that particle size is inversely proportional with sorghum starch digestibility (Al-Rabadi et al., 2009; Tahir et al., 2010); the larger the particles the less digestible they are. Sorghum flour is more digestible than whole sorghum grains because the starch is broken down to smaller

particles, thus increasing the surface to volume ratio. The increased surface area makes it easier for enzymes to break down the starch (FAO, 1995). Another reason that sorghum starch could be difficult to digest could be due to the presence of lipids, presence of protein, presence of minerals, digestion conditions (Al-Rabadi et al., 2009), tannin content, amylose:amylopectin ratio (FAO, 1995), or degree of gelatinization. Light microscopy was used to determine starch granule size.

SAXS on the other hand was used to determine the microstructure of the starch granules. The structure of a starch granule can be defined by four different length scales: the molecular scale (the size of the molecules ~Â), the lamellar repeat distance (8-9 nm), growth rings (120-400nm), and the whole granule morphology (~1µm) (Cameron and Donald, 1993; Pikus, 2005). The combined distance of the mesogen and spacer units give a characteristic peak in SAXS at q=0.63 (1/nm), (Jenkins et al., 1993) in all botanical sources including genetic mutants.

Using the Bragg equation this q-value correlates with a real distance of approximately 10nm. However, Cameron and Donald came up with a mathematical expression that better determines the real distance using six variables: average repeat distance (d), the distance of the crystalline lamellae (Φ), a constant (β), the number of repeats of the crystalline and amorphous lamellae (N), difference in electron density between the amorphous and crystalline lamellae ($\Delta\rho$), and the difference in the electron density between the background and amorphous material ($\Delta\rho_u$) (Cameron and Donald, 1993). The real distance of d was found to be 9nm across all botanical sources (Jenkins et al., 1993). An improved mathematical model was created by Daniels and Donald to account for the distortions of the mesogen layers in potato starch (2003).

Experimental

Sorghum Flour Preparation

Red and White Sorghum grains were separately cleaned with deionized water and let to dry overnight. Rocks and debri were taken out manually. The dried cleaned sorghum grains were then separately milled in a Christy and Norris 8'' Laboratory Hammer mill. The white sorghum resulted in a fine white powder, while the red sorghum resulted in a pink/red powder.

Extraction of Starch Granules

The sorghum starch granules were extracted using a similar protocol to Singh et. al (2010b). 50g of red sorghum flour were measured out. 30ml of distilled water was mixed with the sorghum flour using a mortar and pestle. The resulting wet flour was placed into a 200ml beaker, covered with a damp paper towel, and the beaker was covered with parafilm. Three holes were poked into the top of the parafilm. This process was repeated for white sorghum flour.

Both beakers, with the wetted red and white sorghum flour, were placed into a Binder vacuum oven set at 30°C for one hour. The starch was washed off by mixing the wetted sorghum over a 63µm sieve under a steady stream of deionized water (400ml). The resulting starch slurry was wet sieved twice through the same 63µm sieve to remove impurities (Singh et al., 2010b). The filter cake was then discarded.

The starch slurry was placed in 50ml centrifuge tubes and centrifuged at 4156 RCF, 20°C, for 10 minutes, using a Thermo Fischer Scientific Heraeus Megafuge 16R centrifuge (2011). The supernatant was discarded and the sedimented starch fraction in each tube was purified by suspending in deionized water and centrifuged again. The purification step was repeated four times. The starch was dried in a Binder vacuum oven, at 35°C and 200mbar for 18 hours.

The dried starch was ground with a mortar and pestle and passed through a 63µm sieve to obtain pure starch granules.

Light Microscopy

A purified potato starch was bought from Sigma-Aldrich (33615-250G). A 3% w/v of starch/deionized water was made from each of the three starches: potato, red sorghum, and white sorghum. These starches were mixed using a vibrating stirrer set on high. 15µl of each starch was pipetted onto a separate and cleaned microscope slide. Pictures were taken using a Celeston LCD Digital (model: 44340) Light microscope 100x, and 400x magnification for each starch. Figure 2 displays the pictures of each starch at 100x, and 400x magnification.

Size Distribution of Granules

The size distribution of the granule was found by marking the longest length of fifty granules in CorelDraw using the 100x magnification light micrographs of each starch. The CorelDraw lengths were then calculated into true lengths using the fact that the cross section of the LED screen was 8.89cm. The average and standard deviation of the starch diameters are shown in Table 1.

Table 1: Average diameters and standard deviation of Potato, Red Sorghum, and White Sorghum Starch

Starch Type	Average diameter size (µm)	Standard Deviation (µm)
Potato	26.4	11.9
Red Sorghum	22.9	6.9
White Sorghum	22.9	7.3

It should be noted that this diameter size calculation was very crude. It was notably difficult to determine the beginning and end of the granule, as the boarder to the granule was a thick black line, which is due to the fact that the light microscope does not give an accurate picture due to the granules three dimensional nature.

A histogram of the granule sizes were then made using OriginPro 9 and are shown in Figure 3.

ANOVA analysis was performed using the Tukey test in Origin Pro 9. This analysis showed there was no significant difference in starch granules size among the three different starches. The p-values for all three comparisons were greater than 0.05.

Preparation of Capillaries

A 45% w/v of starch /deionized water was made from each of the three starches. All three starch mixtures were mixed using a vibrating stirrer set on high.

Four disposable glass capillaries (1mm outer diameter) were prepared. The first capillary was filled using a needle with deionized water. The other three capillaries were filled with one of the 45% w/v starch slurries. This concentration was used because it was enough water to gelatinize the starch, but packed enough to prevent sedimentation in the capillary (Cameron and Donald, 1992). Each starch slurry was mixed using a vibrating stirrer set on high right before the capillaries were filled as to ensure the slurry was homogenous.

The end of each filled capillary was then covered in dripping wax from a lit candle. Once the wax was dried the end of the capillary was covered with epoxide (UHU Plus end Fest 300 2-epoxidklieber) to ensure there were no gaps where air could escape. The filled and sealed capillaries were then placed in a 4ºC refrigerator overnight (18 hours).

Scattering Experiments

SAXSpace was used to perform the scattering experiments. A beam of radiation was given off by an X-Ray tube through a line collimated system. The wavelengths of the x-ray beams were between 0.1 and 0.2nm. A Mythen detector was used to gather the scattered radiation information along with SAXS software.

The capillaries were taken out of the refrigerator and allowed to reach room temperature before conducting and SAXS experiments. The parameters for all of SAXS experiments were as follows: SDD=300mm, no absorber, Temp=20˚ C, and high resolution. An empty disposable glass capillary was put into a holder for Hilgenberg glass capillaries with outer diameter of 1mm. The height and rotation was calibrated to h=-1.16mm and rotation=0º to ensure that the beam was hitting the centre of the glass capillary. A scattering experiment was conducted using the empty glass capillary with an acquisition time of 300s and 1 frame.

The disposable glass capillary filled with deionized water was then placed into a stainless steel holder and placed into the SAXS machine. The experiment was started after the pressure reached 5mbar. The room temperature starch filled capillaries were then placed one at a time into the stainless steel holder and the experiment was conducted in the same manner as the water capillary.

The native starch granule capillaries were then gelatinized in a 65˚C water bath. This water bath was set on top of a magnetic hot plate with a magnetic stir bar set at 500 rpm as to create convection current. The starch capillaries were held in the 65˚C bath for thirty seconds, then placed into a room temperature (22˚C) bath for 20s, and then finally a 1.5˚C ice bath for 20 seconds. This was done to prevent the glass capillaries from breaking, but also stop the cooking process. The 30s cooked capillaries were then placed into the stainless steel holder and the SAXS experiment was conducted with the same parameters as stated above. This process was repeated again to the total time that the capillaries were in the 65˚C bath at 60s, 90s, 150s, and 270s. Each time the capillaries were heated for a specified time they were cooled and a scattering experiment was conducted.

SAXS Data Manipulation

All data was calibrate using SAXStreat. The largest peak seen in the beginning of the data was set to 0. The data was then normalized in SAXSquant. The background scattering from the empty capillary was first was subtracted both from the sample and the water measurement. Secondly, the volume fraction weighted pure water scattering, I_{water}, was subtracted from the pure sample scattering, I_{sample}. The volume fraction of water in the sample was 0.55. Thus, the pure starch scattering, I_{starch}, is:

Equation 1: Background Subtraction:

$$I_{starch} = I_{sample} - 0.55 * I_{water}$$

The background scattering coming from the capillary and the water was subtracted to display the scattering only from the starch. All starch files were then desmeared.

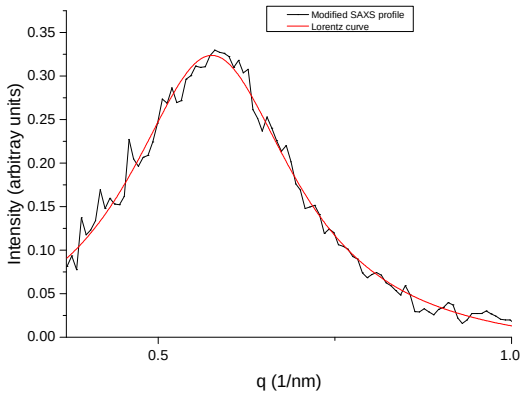

Figure 1: Lorentz fit of native White Sorghum starch granule peak corresponding to lamellar repeat distance

OriginPro 9 was used to find the peak distances that correspond to the lamellar repeat distance for each native starch. First an exponential decay function was found so that the decay function hit the peak at both minima. The exponential decay function was then subtracted from the original native granule data set. A new graph was made using the subtracted data set from q=0.2 (nm^{-1}) to q=1.0 (nm^{-1}). A best fit non-linear Lorentz peak was fit to this curve. The sorghum curves were normally distributed.

However, the potato starch curve was asymmetrically distributed. An asymmetric curve and a Gaussian curve were fit to the potato starch curve. However, the best fit curve for the native potato starch was determined by a smaller parameter set (q=0.4-0.7 nm^{-1}) using the Lorentz fit.

The Lorentz fit process was repeated for every curve through the heating process. Figure 1 shows an example of the Lorentz fit to native White sorghum. This was done to analyse the change in peak height, which may be proportional to the resistance of gelatinization. The change in peak height over tune in a 65˚C bath is shown in Figure 6.

Potato starch also exhibited a peak in the range of q=0.3 to q=0.5 nm^{-1}. The exact q value of this peak was found using the same procedure in OriginPro 9 as previously stated.

Results

Light Microscopy

Potato starch and sorghum starch granules have a vastly different size and shape as shown in Figure 2. Potato starch is elliptical or spherical in shape, while both red and while sorghum are polyhedral.

The size of starch granules are inversely proportional to the rate constant of α-amylase (Al-Rabadi et al., 2009). The size distribution of the starch granules are displayed in histograms in Figure 2. However, as indicated in Table 1, the average diameter of potato starch is 26.4 µm, while both Red and White sorghum have an average diameter of 22.9 µm. The standard deviation for potato starch is however, quite large (11.9 µm). 18% of the potato granules have a diameter >40 µm, while Red sorghum has 0% and white sorghum has only 2%.However both red and white sorghum granules were not significantly smaller than the potato granules as indicated by ANOVA analysis in Origin Pro 9.

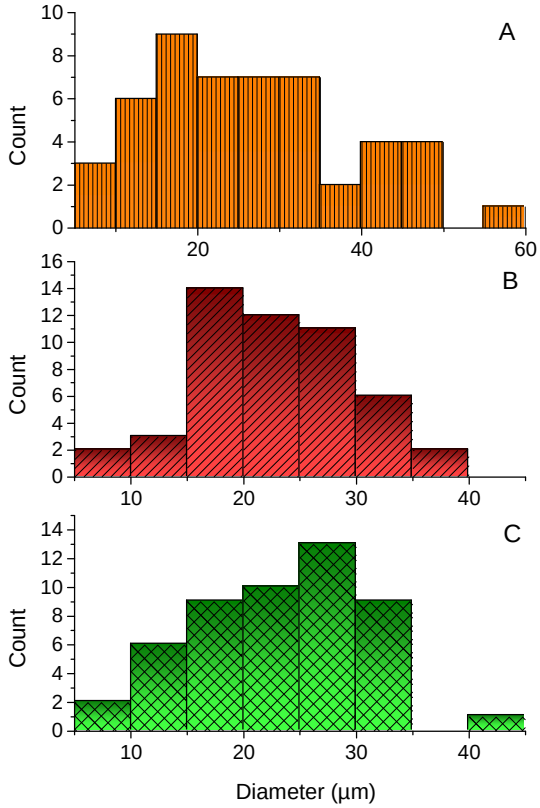

Figure 3: Histogram of starch granule diameters of A) Potato, B) Red Sorghum C) White Sorghum

Although there were some potato granules that were much larger than the sorghum granules, the standard deviation indicates that there is no significant difference in size of the granules, and thus the diameter may not be the causal factor as to why sorghum is less digestible than potato starch.

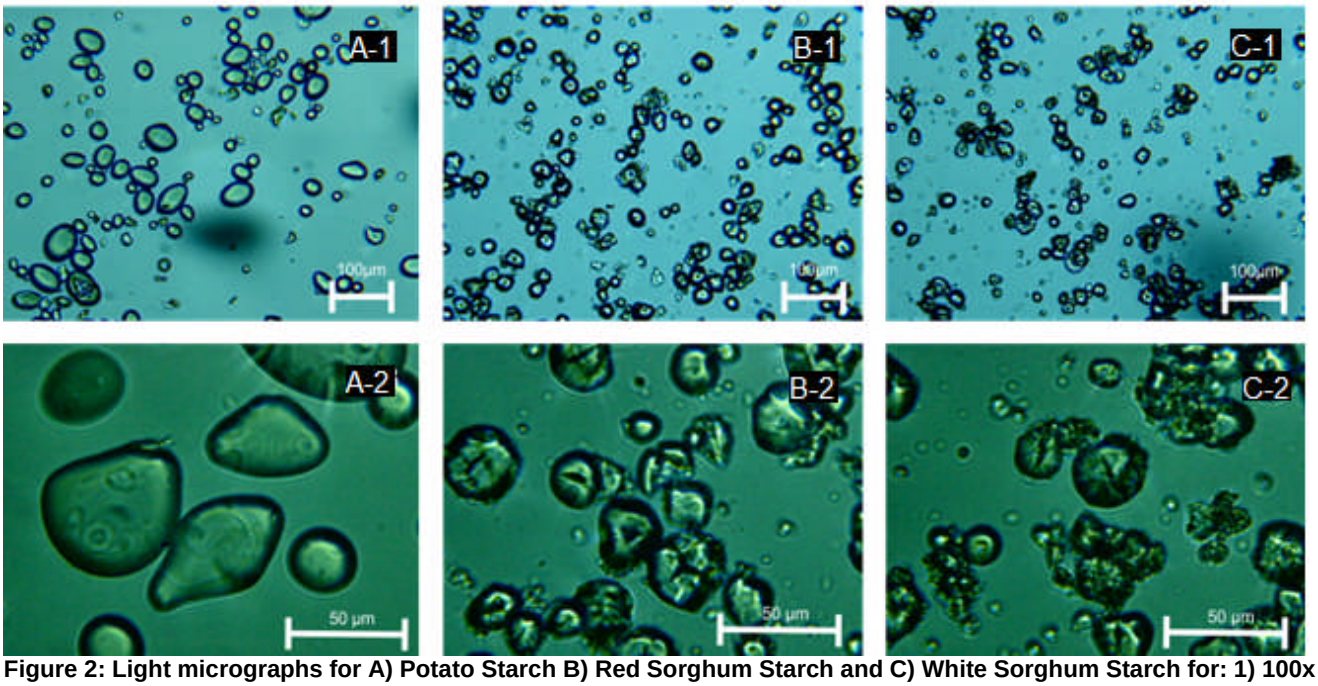

Figure 2: Light micrographs for A) Potato Starch B) Red Sorghum Starch and C) White Sorghum Starch for: 1) 100x and 2) 400x magnification

SAXS Interpretation

Native starch granules show a peak at approximately q=0.63 (1/nm) according to Cameron and Donald (1992). This peak is related to the lamellar repeat distance, which has been shown to be the 9nm across varying botanical sources (Jenkins et al., 1993). However, the q value of the peaks for both red and white sorghum were only 0.58 (1/nm) and 0.57 (1/nm) respectively. Figure 4 A) shows a close up of the peak shown in potato, red sorghum, and white sorghum native starch granules.

It is important to note that, as described by Jenkins et. al, that the q value does not directly correspond to the lamellar repeat distance (1993). This is because the peak shown by starch is too broad. A more sophisticated mathematical structure, as shown by Daniels and Donald, should be used to determine the lamellar repeat distance (2003). However, for the context of this report, the q values and corresponding Bragg distances, as determined by Equation 2 are reported in Table 2 to show a difference between the potato starch and the sorghum starch peak distances.

Equation 2: Bragg equation

$$d = \frac{2\pi}{q}$$

The Standard error for q was reported with the Lorentz fit in Origin 9 and the standard error for the Bragg value of *d* was calculated using Equation 3. The standard deviation of q shows that there is a significant difference in q values between all three starches.

Equation 3: Standard error of *d*

$$\Delta d = d\left(\frac{\Delta q}{q}\right)$$

It is evident in that both red and white sorghum have a significantly smaller q distance than potato starch as shown by error analysis. The smaller q value for red and white sorghum translates to a significantly larger Bragg value. A larger *d* spacing could implicate that the mesogen or the spacer units are larger. If the mesogen layer is larger, this could implicate a larger amount of crystallinity, and thus amylopectin. However, when all other variables are considered (Φ, β, N, $\Delta\rho$, and $\Delta\rho_u$), the lamellar repeat distance may be the exact same, 9 nm, as indicated by Jenkins et al. (Jenkins et al., 1993).

Table 2: q values and Bragg values

Starch Type	q value (nm^{-1})	Bragg value (nm)
Potato	$0.62 \pm 2.0 \times 10^{-3}$*	10.1 ± 0.033*
Red Sorghum	$0.58 \pm 8.9 \times 10^{-4}$*	10.8 ± 0.017*
White Sorghum	$0.57 \pm 1.3 \times 10^{-3}$*	10.9 ± 0.025*

* Indicates significant difference for values of the same row

One interesting point in Figure 4 B), is that potato starch shows a distinct and broad peak at a q=3.89 ± 0.002 nm^{-1} while neither red or white sorghum showed any peak in that range. Using the Bragg equation the real distance of this peak is 1.61 ± 7.0×10^{-4} nm^{-1}. This peak could correspond to the type of crystallinity of the starch. Further ongoing mathematical analysis should indicate why red and white sorghum show no peak in this range.

The structure of native starch granules is important to understand the physiochemical properties of that starch. However, as most all starch is consumed as a gelatinized starch, the structure of gelatinized starch granules was studied in order to see if there was any difference in how the sorghum starch was gelatinized vs. potato starch.

The q value of the peak remained the same over all the SAXS profiles for each starch over time in a 65˚C bath, which has been corroborated by

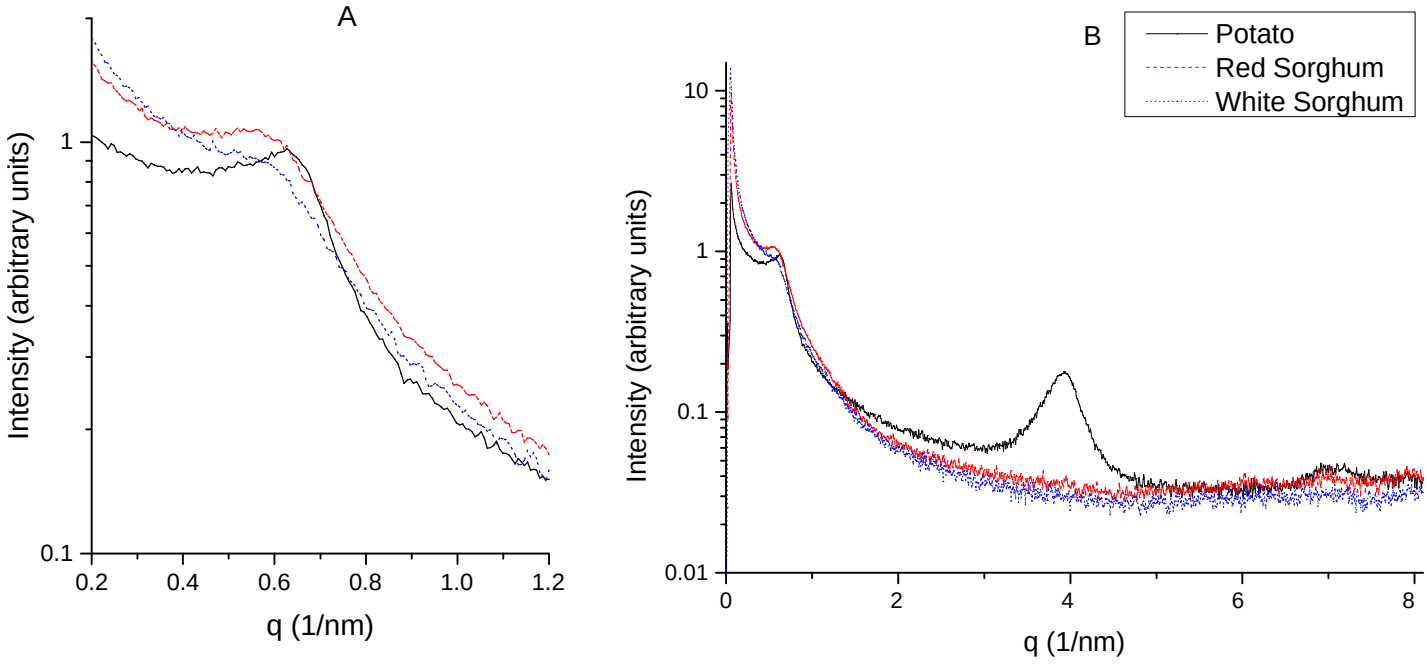

Figure 4: A) Partial SAXS profile and B) full SAXS profile of 45% suspensions of native Potato, Red Sorghum, and White Sorghum starch, at room temperature

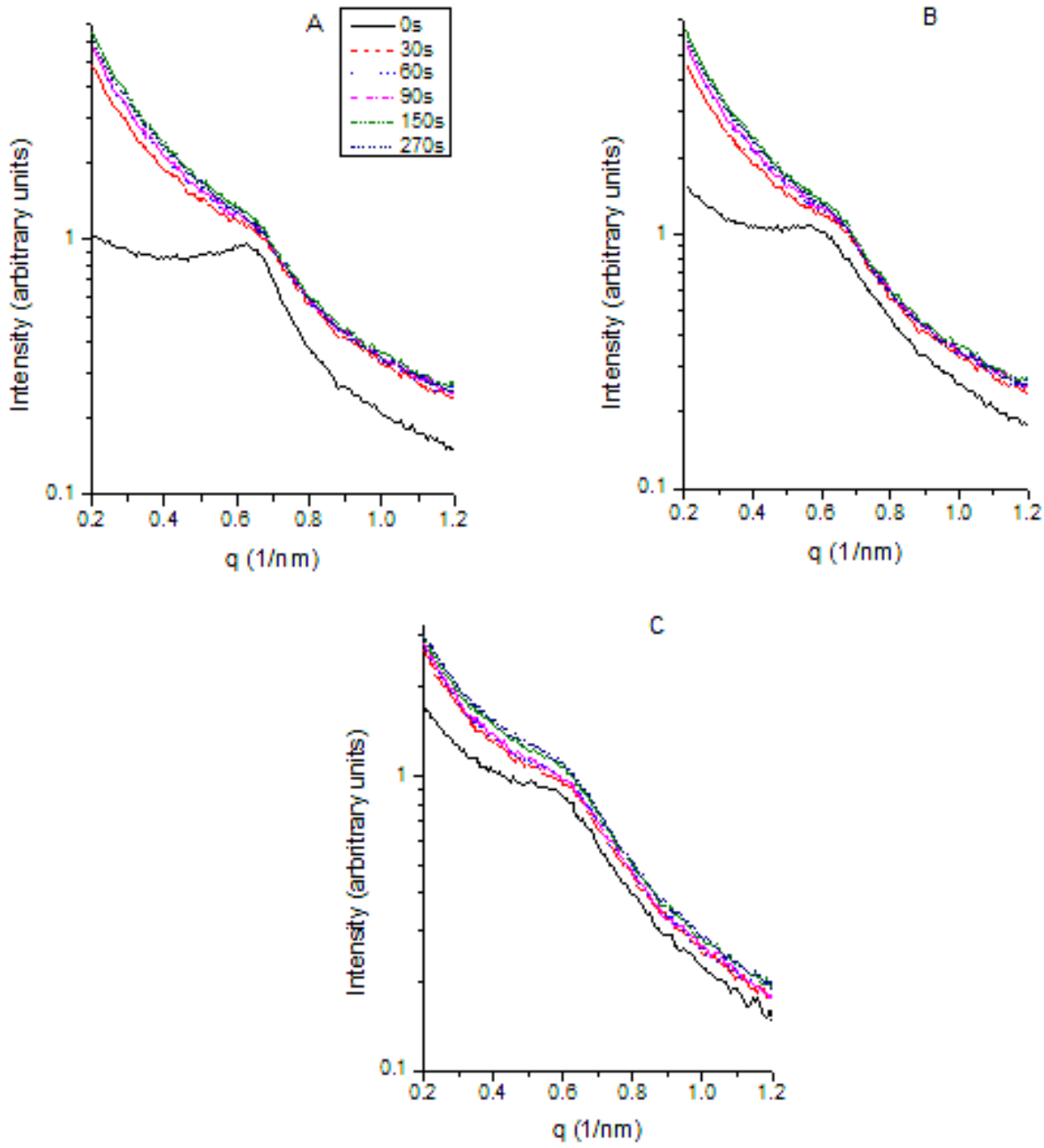

Figure 5: Partial SAXS profile of A) Potato Starch, B) Red Sorghum, and C) White Sorghum at varying time scales: 0s, 30s, 60s, 150s, 270s in a 65° C water bath

Cameron and Donald (1992). Gelatinization does not change the peak distance, rather it should diminish the strength of the peak as indicated in Figure 5 for all three starches.

The height of the curve for every phase in gelatinization was taken from the Lorentz curve fit to each stage. The change in height is displayed in Figure 6. Potato Starch shows the largest change in height from 0s to 30s, while white sorghum shows the least amount of change. In fact, the height of the peak increased over time for white sorghum. Red sorghum on the other hand showed a similar change in peak height to potato starch from 0s to 30s, but it was less pronounced. This is evident in the SAXS profiles displayed in Figure 5.

The potato starch has the largest change in peak height over time. This may indicate the potato starch has a higher amount of amylose, as amylose is more easily disrupted from gelatinization.

The increase in peak height for white sorghum may indicate more resistance to gelatinization and an higher amount of amylopectin which is corroborated by the *d* spacing Bragg valued.

Most starch is consumed in a gelatinized state rather than a native state because gelatinized helps digestion. Although amylopectin is more easily susceptible to amylolytic attack because of its branched nature, if amylopectin is not fully gelatinized, its tight crystalline nature may prevent amylolytic attack and prevent full digestion in red and white sorghum as compared to potato starch.

It is also impetrative to note that the SAXS profiles of the gelatinized starch were completed after the starch had cooled. Amylose will retrogradade

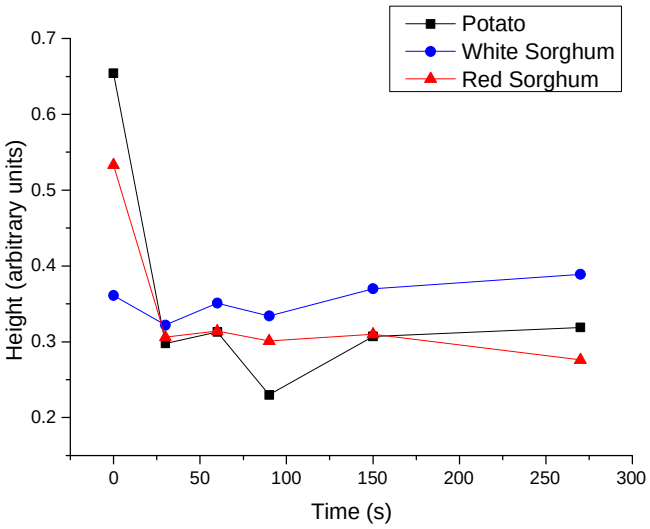

Figure 6: Change in peak height over time in a 65˚C for Potato, Red Sorghum, and White Sorghum Starches

upon cooling to room temperature. This could affect the crystallinity of the starch, and thus affect the peak height.

Discussion

This study aimed to ascertain information from SAXS and light microscopy to review the physiochemical properties of sorghum starch. These properties could lead to a better understanding as to why sorghum has a higher amount of resistant starch, and is thus harder to digest.

Light microscopy showed that the control, potato starch contained elliptical and spherical starch granules, while red and white sorghum granules were polyhedral. Through ANOVA analysis there was no significant difference in the means of the starch granule size. Although starch granule size has been shown to be inversely proportional to digestibility, this may only be comparable among one type of starch. For example, it has been shown that sorghum grains are harder to digest than sorghum flour. Starch granule size may not be the leading cause of indigestibility of sorghum starch. Further digestion experiments should be performed to analyse this more closely.

Although no conclusion can be made about the true *d* spacing of the starches, the significant difference in the q values are intriguing. The Bragg equation indicated that there was a significant difference in *d* spacing among all three starches with a distance of 10.1 ± 0.033, 10.8 ± 0.017, 10.9 ± 0.025, for potato, red sorghum, and white sorghum starch respectively.

Although there is a significant difference in *d* spacing, a more complex mathematical model as developed by Daniels and Donald should be used to get a clearer idea of the physiochemical parameters of red and white sorghum.

Further gelatinization experiments should be completed for a longer period of time. This could be completed within the SAXS machine so that retrogradation of amylose does not affect the scattering profile.

In addition, tradition methods of cooking sorghum use fermentation. Fermentation could affect the crystalline structure of sorghum by breaking apart amylose and amylopectin into respective smaller polysaccharides or glucose units. The SAXS profiles of fermented sorghum could shed light to the structure of this more easily digestible method of cooking.

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