IN VITRO DIGESTIBILITY OF STARCH IN SORGHUM DIFFERING IN ENDOSPERM HARDNESS AND FLOUR PARTICLE SIZE

by

XIAOLI XU

B.S., Biotechnoloy, Zhejiang University, 2003
B.S., Computer Science, Zhejiang University, 2003
M.S., Oncology, Zhejiang University, 2006

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Approved by:

Major Professor
Dr. Yong-Cheng Shi

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Abstract

In vitro digestibility of starch in sorghum grains differing in endosperm hardness and flour particle size was assayed by an Englyst resistant starch (RS) method. The starch digestibility increased as the particle size of flour decreased, but no significant difference in starch digestibility was observed among sorghum flours milled from grains with different hardness. To further understand the digestion of starch in sorghum, the effects of protein on starch digestion and amylose content in starch were determined. pH value was a factor affecting protein digestion since protein digestibility was higher at pH 2.0 than that at pH 1.3. Protein hydrolysis increased with time of pepsin treatment, leading to an increased starch digestion. RS content was 10.61-29.54% in native sorghum flours and 8.47-26.28% in isolated sorghum starch. The amounts of γ-kafirins extracted increased with time of pepsin treatment while α- and β-kafirins decreased. The starch in sorghum flour with median hardness had a higher amylose content (23.9%) than the starch in hard and soft flours (~21%), which gave lower starch digestibility. Protein digestibility decreased after cooking while starch digestibility increased. Sulfhydryl groups decreased after cooking, indicating that disulfide bonds formed between protein molecules and may have formed a barrier for enzymes to access and digest starch. Confocal laser scanning microscopy (CLSM) showed that the protein matrix was less evident after pepsin treatment. As a result, starch digestion increased after protein matrix was removed.

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CHAPTER 1 - INTRODUCTION

STARCH DIGESTION PROPERTIES

Starch is classified into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) according to the rate of glucose release and its absorption in the gastrointestinal tract (Englyst et al, 1992). RDS is the portion digested within 20 minutes and SDS is digested between 20 and 120 minutes.SDS is believed (Englyst et al, 1992) to be slowly but completely digested, leading to a slower entry of glucose into the blood stream and lower glycemic response. RS can not be digested in the small intestine and is left in the colon. Englyst et al (1982) defined RS as that starch that remained after enzymic hydrolysis, resists digestion in the stomach and small intestine, and ferments in the large intestine. The concept was improved by EURESTA (Asp, 1992) as the total amount of starch, and the products of starch degradation that resists digestion in the small intestine of healthy people. The amount of RDS is positively correlated with the glycemic index (GI) of food products (Englyst et al, 1999). It has been suggested that the content of RDS and SDS can be used to predict the GI of cereal-based food products (Englyst et al, 2003).

GI represents the level of the postprandial glucose rise in blood as compared to a reference food or glucose (Jenkins et al, 1981). Long-term intake of foods with a high GI has been shown to be associated with obesity and related chronic diseases of diabetes and cardiovascular disease (Ludwig, 2000). Dietary carbohydrates, such as starch, effect on human health are important, because they provide 45-65% of the total caloric intake (Dietary Guidelines for Americans, 2005). Thus, starch in food is important for healthy diets, and the starchy food with less refined and less processed should be increased in the diet since it leads to a low GI value.

The digestion process of starch is catalyzed by amylolytic enzymes which are comprised of pancreatic α-amylase and the intestinal brush border glucoamylases, maltase-glucoamylase, and sucrose-isomaltase (Nichol et al, 2003). The activities of these enzymes affect the rate of starch digestion. SDS is slowly digested and is related to its substrate property. Ferguson et al (2000) revealed that some native cereal starches with semicrystalline A-type structure contain high levels of SDS, more than 50% in maize and sorghum starches. A number of studies on raw

cereal starches showed that the slow digestion property is affected by their biosynthesis (James et al, 2003), structure (Buleon et al, 1998 and Tester et al, 2004), physicochemical properties (Oates, 1997), and enzymatic hydrolysis (Tetlow et al, 2004). Zhang et al (2006a) reported the side-by-side digestion mechanism and layer-by-layer digestion pattern of slow digestion property of native cereal starches. The crystalline and amorphous regions of starch granules were evenly digested through a mechanism of side-by-side digestion of concerntric layers of semicrystalline shells of native starch granules. Enzymatic hydrolysis requires the binding of amylolytic enzymes to starch molecules.

After slow digestion, RS is left in the colon and fermented by colonic bacteria. Hence, RS has potential for prebiotic applications and has physiological benefits which are associated with disease prevention. Fermentation of RS produces short-chain fatty acids (SCFA), such as acetate, propionate, and butyrate. These products lower the overall pH of the colon, induce chemoprotective enzyme activity, and hinder growth of harmful colonic bacteria. Thereby, RS plays a role in protecting against colorectal cancer (Burns and Rowlands, 2000; Ferguson et al, 2000; Topping and Clifton, 2001; and Wollowski et al, 2001). Other benefits of RS consumption include lowering of plasma cholesterol and blood lipids, as well as improved glucose tolerance (Vanhoof and De Schrijver, 1998 and Voragen, 1998).

STRUCTURE AND CHEMICAL BASIS OF SORGHUM FLOUR

Sorghum is one of the most important cereals in the world (Hamaker et al. 1986) and the main food for people that live in the semi-arid tropics of Africa, Asia and South America because of its tolerance to drought conditions (Elmalik, 1985). It is known that, among the cereals, the starch in sorghum flour is relatively low in digestibility (Rooney and Pflugfelder, 1986 and Elkin et al, 2002). Thereby, sorghum could therefore be a potential source of RS. Different digestibilities depend on different cereal sources (Aarathi et al, 2003), indicating the intrinsic factors could be the nature of cereals themselves, such as compositions and structure. It is suggested that low starch digestibility in sorghum is associated with structure of sorghum flour (Aarathi et al, 2003).

ENDOSPERM CHARACTERISTICS

The endosperm of sorghum is composed chiefly of storage parenchyma cells filled with starch granules embedded in a continuous matrix of protein (Watson et al, 1955). It contains

regions of both floury and horny endosperm. Starch is the major component of sorghum flour. According to the type of starch in their endosperms, the sorghum grains can be classified into waxy and non-waxy. Starch consists of amylose, a linear glucan with α-1,4 linkages, and amylopectin, a highly branched glucan with α-1,4 and α-1,6 linkages. Non-waxy sorghum contains starch that is composed of approximately 25% amylose and 75% amylopectin, whereas starch in waxy sorghum is almost 100% amylopectin (Sikabbubba, 1989). Non-waxy sorghum starch is highly resistant to enzymatic digestion, whereas waxy starch is highly susceptible to enzyme digestion (Sullins and Rooney, 1975). In non-waxy sorghum flour, there is a high concentration of protein bodies in the peripheral endosperm area, while the protein bodies are more evenly distributed in waxy sorghum flour (Fig. 1.1).

The higher the content of amylose, the lower is the digestibility of starch (Aarathi et al, 2003). Previous study have shown that there are apparent differences in the digestibility of amylose and amylopectin (Goddard et al, 1984). The possible reason is that amylopectin has a larger surface area and is highly organized, leading to the formation insoluble aggregates (Aarathi et al, 2003).

Non-waxy

Waxy

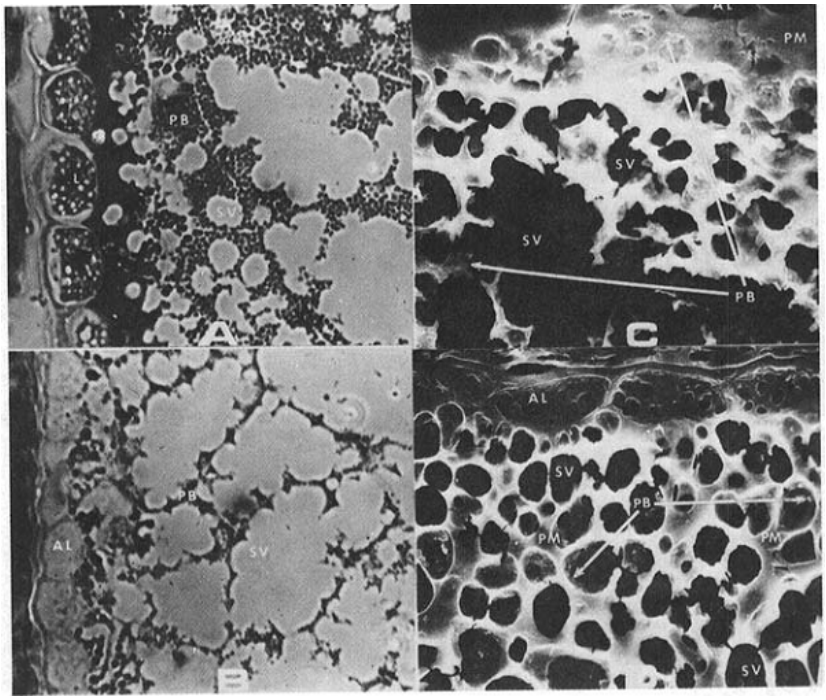


Figure 1.1 Light and scanning electron photomicrographs comparing the endosperm structure of non-waxy Kafir and waxy Kafir (Sullins and Rooney, 1975). Left, peripheral endosperm; right, central endosperm.

STARCH GRANULE ORGANIZATION

Buleon et al (1998) demonstrated that starch granule organization has different levels of structure related to enzymatic hydrolysis: granular structure, superamolecular structure, and molecular structure. Granular structure mainly includes shape, size, and porosity. There are pores and channels within starch granules. Porosity is considered as the basis for their inside-out digestion patterns. Channels are the main route of enzyme penetration and the central cavity area is the starting point of enzyme digestion (Benmoussa et al, 2006). For example, in a sorghum mutant with relative high protein digestibility, the channel density of isolated starch is more pronounced than found in normal wild type lines. Fig. 1.2 shows the changes of pores and channels during digestion. These authors also found a collapsed "doughnut-shaped" structure in isolated starch from a unique sorghum mutant and the granules were digested rapidly, which indicates that amylases appeared to have fast access to the collapsed-appearing starch granules.

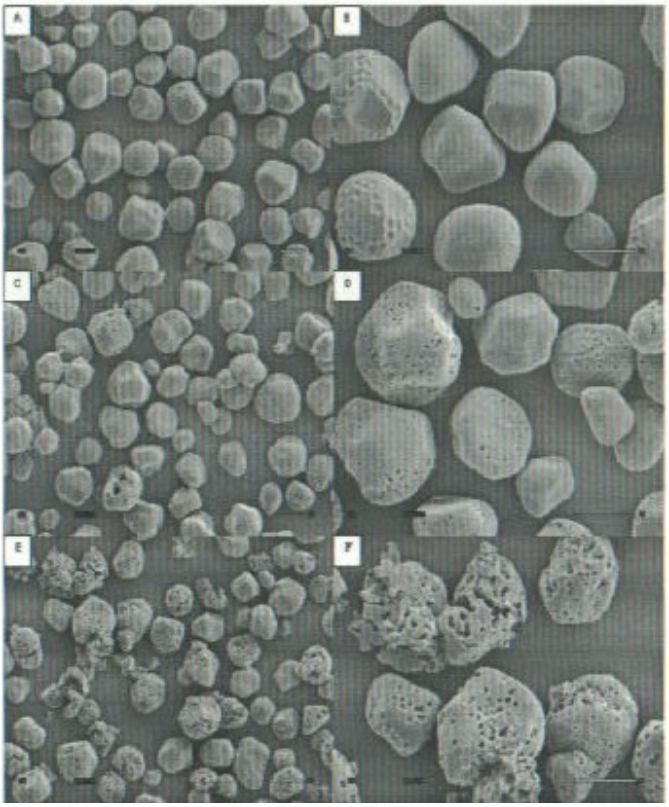


Figure 1.2 Scanning electron micrograph of isolated starch from normal sorghum four (Benmoussa et al, 2006). A & B are undigested sorghum flour; C & D are sorghum flour after 30 min digestion; and E & F are after 1 hour digestion. A, C, and E are 1000× magnification, while B, D, and F are 2500× magnification.

The supramolecular structure includes the crystalline type, perfection of crystallites, degree of crystallinity, the arrangement of crystalline and amorphous materials, which are called growth rings, and organization of crystalline and amorphous lamellae in the hard shell of the growth rings (Buleon et al, 1998). The growth rings are arranged in concentric layers from the center to the surface of the starch granules. Depending on the source of starch, different levels of structure may govern the hydrolysis pattern and rate of enzyme reaction. For example, both crystalline type and the surface property of potato starch are associated with its resistance to α-amylase hydrolysis, whereas the supramolecular structure is normally the major controller for enzymatic hydrolysis of native cereal starches (Oates, 1997).

The molecular structure includes the fine structure of amylopectin and amylose. Amylopectin is the organizer of starch granules and the major component in normal cereal starches, so its fine structure determines the crystalline type and its perfection in native starch granules.

PROTEIN MATRIX AND COMPOSITION

There is a protein matrix encapsulating the starch granules in flour (Fig. 1.3). Sullins et al (1971) reported that the increased feedlot efficiency of reconstituted sorghum grain fed to steers was directly related to the partial breakdown to the protein matrix, especially that in the peripheral endosperm area of the kernel. The reason is that the accessibility of starch to hydrolysis by glucoamylase was influenced by the protein matrix (Lichtenwalner et al, 1978). Protein composition of the protein matrix is important to understand this influence.

Kafirins (sorghum prolamins) are the most abundant protein in sorghum, making up ≈70–80% of the total endosperm protein (Hamaker et al, 1995). They have been classified according to structure, molecular weight, and solubility characteristics into α-(MW 25,000 and 20,000), β-(MW 20,000, 18,000, and 16,000), and γ-kafirins (MW 28,000) (Shull et al, 1991). α-Kafirin is the major sorghum storage protein, making up ≈80% of total kafirins and ≈60-70% of the total protein in the endosperm (Watterson et al, 1993). α-Kafirin is enclosed in protein bodies surrounded by γ-kafirin, and to a lesser extent β-kafirin. The amino acid compositions of β- and γ-kafirin are ~ 5 and 7 % cysteine, respectively, which makes them unique (Shull et al, 1991). Among the sorghum proteins, γ-kafirin is highly disulfide-bound in mature grain, and was found to be more resistant to digestion than α-kafirin, especially after cooking (Oria et al, 1995), whereas α-kafirin is highly digestible both before and after cooking (Hamaker, unpublished data,

from Aboubacar et al, 2001). It was suggested that γ-, and to a lesser extent β-kafirins, form a disulfide-bond enzyme-resistant layer at the periphery of protein bodies that restricts access by proteases to the easily digestible α-kafirin (Choi et al, 2008). Fig. 1.4 shows the difference between protein bodies structure of normal and high-digestibility sorghum line. Oria et al (2000) conducted immunocytochemistry analysis and found the similarities in localization of α- and β-kafirins within the protein bodies of high-protein digestibility and wild-type lines. However, in the high protein digestibility lines, γ-kafirin was located at the base of the folds of the protein bodies instead of at the periphery, as is characteristic of wild-type lines.

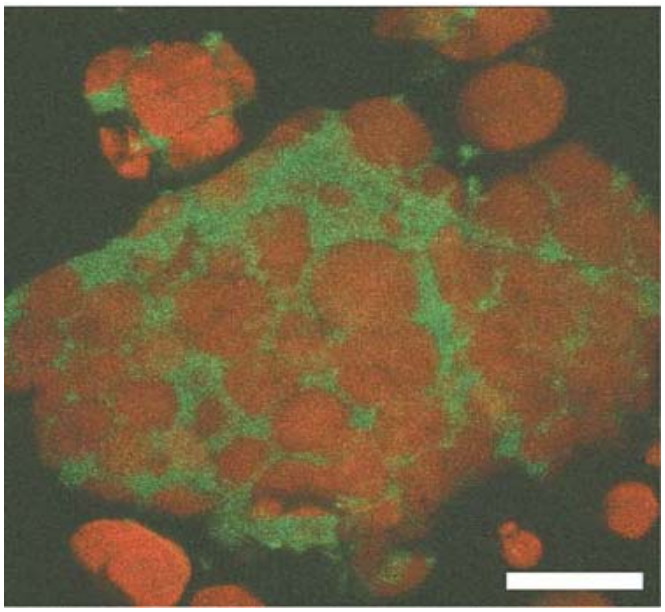


Figure 1.3 Confocal laser scanning micrographs of sorghum floor, green: protein, dark orange: starch (Choi et al, 2008).

During development, kafirins are synthesized and deposited inside the rough endoplasmic reticulum to form protein bodies. Within the protein body, the kafirins are distributed in a nonhomogeneous fashion. Immunocytochemistry showed that α-kafirin is located in light-staining areas mainly in the interior of the protein body, and β- and γ-kafirin are found in dark-staining areas inside and at the periphery of the protein body (Shull et al, 1992). Protein have been found to line the channels that lead into the interior of sorghum granule (Han et al, 2005). Those proteins could interfere with inward migration of α-amylase during digestion.

Enzymatic hydrolysis of starch granules is a solid-solution two-phase reaction in which the enzyme needs first to diffuse toward and bind to the solid substrate, and then to cleave the glycosidic linkages (Zhang et al, 2006b). Thus, the accessibility to the protein matrix affects the reaction rate by enzyme diffusion. However, the mechanism of protein matrix affecting digestion of starch under different cooking conditions is not well understood.

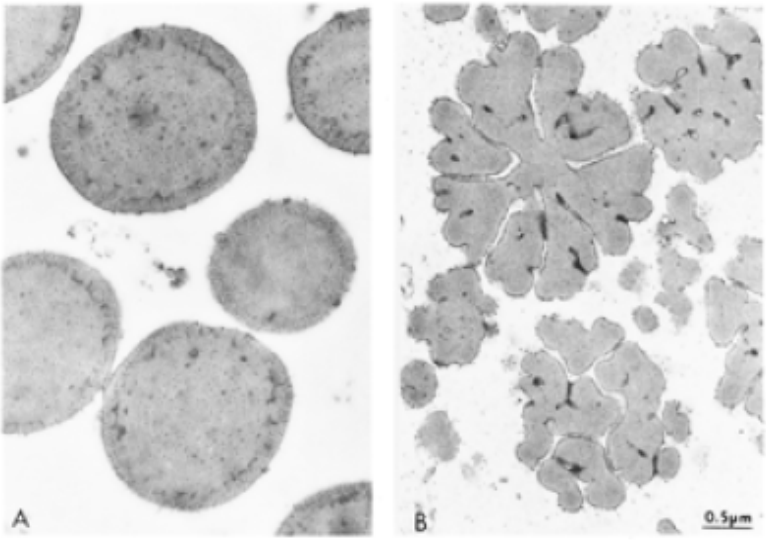


Figure 1.4 Protein bodies structure of wild-type (A) and high-protein digestibility (B) sorghum lines (Oria et al, 2000).

FACTORS AFFECTING SORGHUM STARCH DIGESTIBILITY

It is known that starch digestibility is affected by the plant species, the extent of starch-protein interaction, inhibitors, the physical form of the granule, and the type of starch (Rooney and Pflugfelder, 1986). Tannins are the inhibitors of sorghum starch digestion. Chibber et al (1980) reported that the presence of tannins lowered the digestibility of sorghum grain. Prolamins (i.e., the kafirins), alcohol soluble proteins, were found to lower sorghum starch digestibilities when in high amounts in the grain (Axtell et al, 1981).

As discussed in the previous section, any chemical or enzyme which has effect on the disintegration of protein matrix could increase starch digestion. For example, sodium bisulfite can cleave intra- and intermolecular disulfide bonds and thus disrupt the protein matrix (Fig. 1.5), so starch digestion is improved after treatment with 10 mM sodium bisulfite (Choi et al, 2008).

Pepsin has a similar effect by hydrolyzing the protein matrix. There is a lower amount of protein in flour after 2-hour pepsin treatment than before (Fig. 1.6).

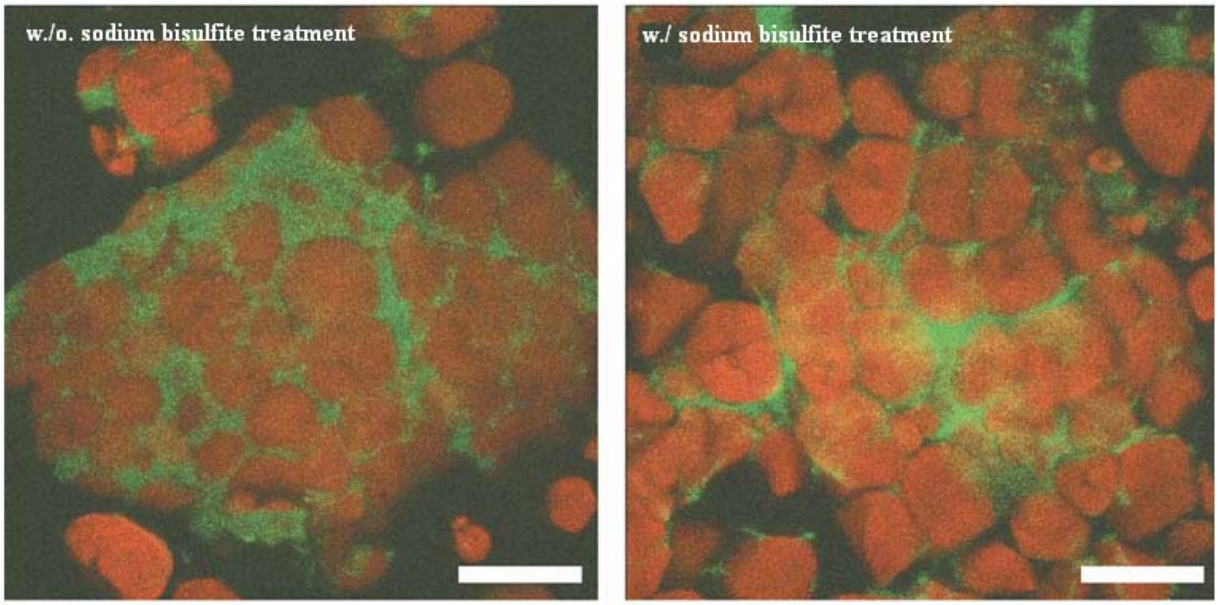


Figure 1.5 Comparison of protein matrix before and after sodium bisulfate treatment, green: protein, dark orange: starch (Choi et al, 2008).

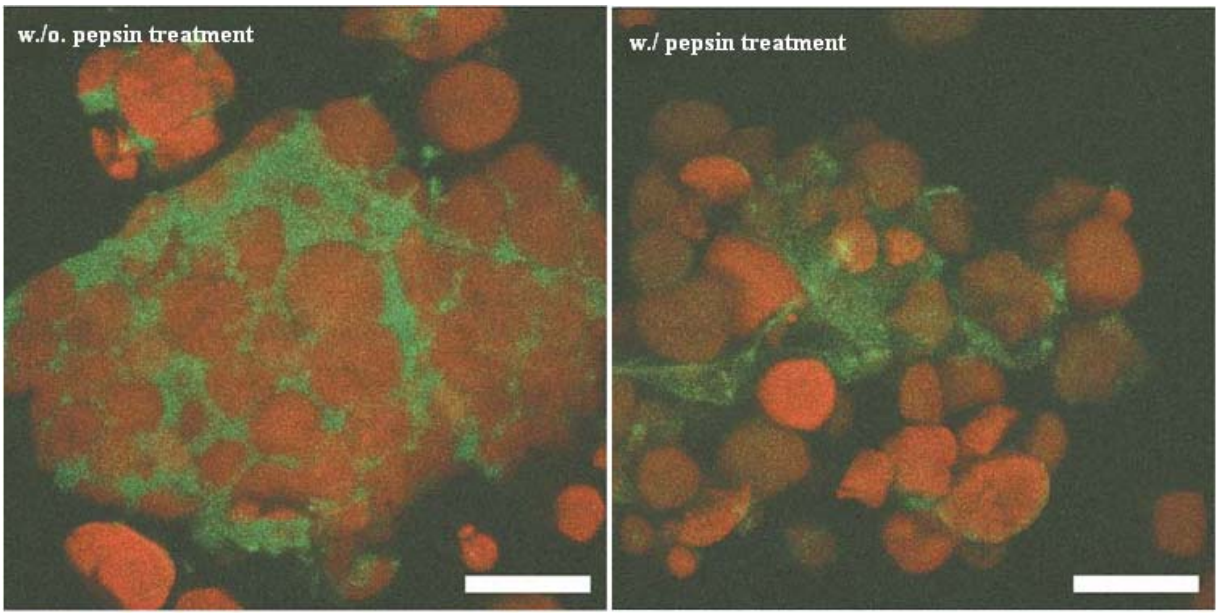


Figure 1.6 Comparison of protein matrix before and after pepsin treatment for 2 hr, green: protein, dark orange: starch (Choi et al, 2008).

Processed grain has been demonstrated to improve feed utilization and nutrients (Theurer, 1986). Sorghum is often for processed before being used as a feed. The processing methods include steam-flaking, early harvest ensiling (high moisture), popping, exploding, roasting or micronizing, and fermentating (Hale, 1980). Processing methods employ proper combinations of moisture, heat and pressure, in order to improve the rates of *in vitro* amylolytic attack of starch in cereal grains by both ruminal microbial and pancreatic enzyme sources (Theurer, 1986). Kernel hardness, kernel size and rate of water uptake are among the important factors (Rusnak et al, 1980).

Cooking conditions are studied most among all the factors affecting sorghum starch digestibility. Cooking of cereal starches brings about certain changes, e.g., physical and chemical disruption, gelatinization of starch granules, and protein matrix collapse (Aarathi et al, 2003). Other physicochemical factors have been advanced as possible causes of the observed restricted protein and starch digestibility in sorghum. The most important factor seems to be cross-linking of the storage proteins (kafirin) in the endosperm (Zhang and Hamaker, 1998; Duodu et al, 2003; Ezeogu et al 2005).

The extent of starch gelatinization is dependent on the amount of water present, cooking time and temperature (Leach, 1965 and Williams and Bowler, 1982). The little and more slowly the starch swells, the less the starch gelatinization occurs (Davis, 1994). Chandrashekar and Kirleis (1988) reported the effect of protein on limiting starch gelatinization in sorghum. It was suggested that the presence of protein bodies around starch granules may restrict granule swelling and starch gelatinization and as a result reduce the susceptibility to enzymatic attack (Aarathi et al, 2003). This may be partially responsible for the low digestibility. Also, the protein cross-linking involves the cysteine-rich γ- and β-kafirin species at the protein body periphery (Oria et al, 1995), which could impede the starch granule gelatinization and the subsequent digestibility (Chandrashekar and Kirleis 1988; Zhang and Hamaker 1998; Ezeogu et al 2005). X-ray diffraction patterns found that there is A-type pattern in raw starch with typical peaks which was not shown in cooked starch (Shin et al, 2004). It is mostly due to the fact that cooked starch is mainly composed of amorphous regions.

Both starch hydrolysis (Ezeogu et al, 2005) and protein hydrolysis (Duodu et al, 2002) are restricted in sorghum, especially when the grain is wet cooked.

Increased energy of cooking caused the collapse and matting of the sorghum vitreous endosperm matrices (Ezeogu et al, 2008). Fig. 1.7 shows the collapse of protein matrix after cooking.

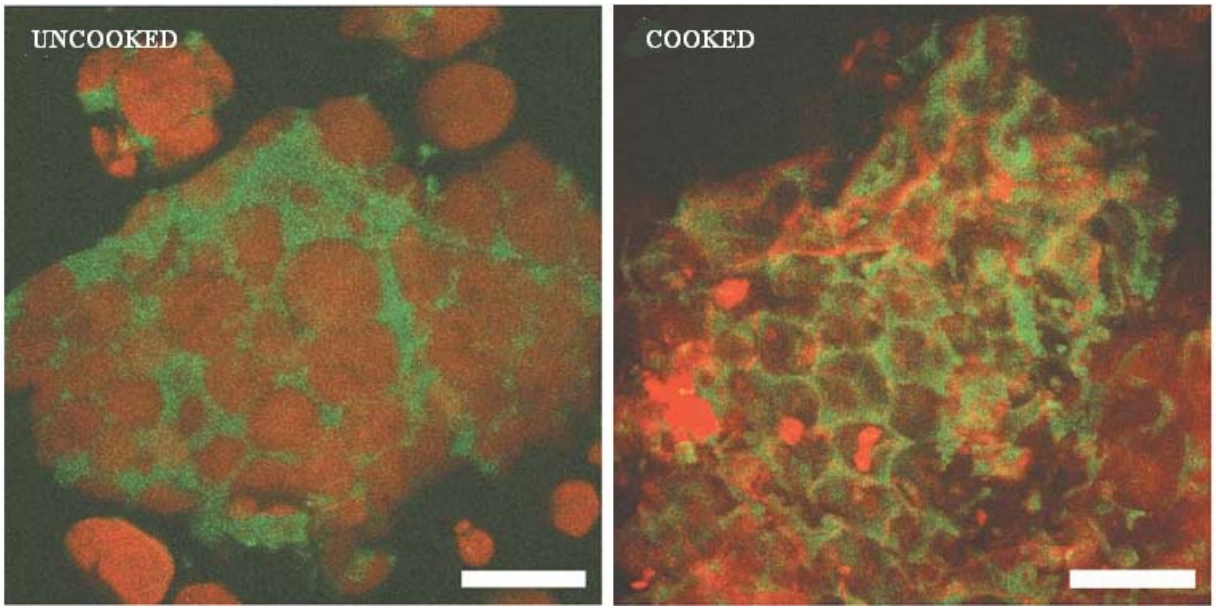


Figure 1.7 Comparison of protein matrix before and after cooking, green: protein, dark orange: starch (Choi et al, 2008)

Disulfide bonding and an increase in β-sheet structure of protein occurred with cooking and at the same time, disulfide bonding increased and was the greatest in the vitreous endosperm (Ezeogu et al, 2008). Because an increased disulfide-bonded protein matrix limits the expansion of the starch granules, amylase access is limited, too. As a result, cooked sorghum flour has low starch digestibility compared to cooked maize flour (Ezeogu et al, 2008). When sorghum was cooked, the matrix of a hard endosperm type appeared as convoluted (weblike or sheetlike) sheets with protein bodies buried in the matrix (Chandrashekar and Kirleis 1988). The starch in vitreous endosperm was hydrolyzed more slowly than that in floury endosperm (Ezeogu et al, 2005). In contrast, the effect of cooking is different in the floury endosperm in that the protein matrices expanded and broke up to some extent. The protein matrix of a soft endosperm sorghum expanded to a greater degree and gave a more open structure on cooking (Chandrashekar and Kirleis 1988). These effects were a consequence of expansion of the starch granules through water uptake during gelatinization (Hamaker and Bugusu, 2003).

If sorghum flour has already been treated with chemicals or enzymes to disintegrate the protein matrix, the effect of cooking is more interesting. Oria et al (1995) used electron

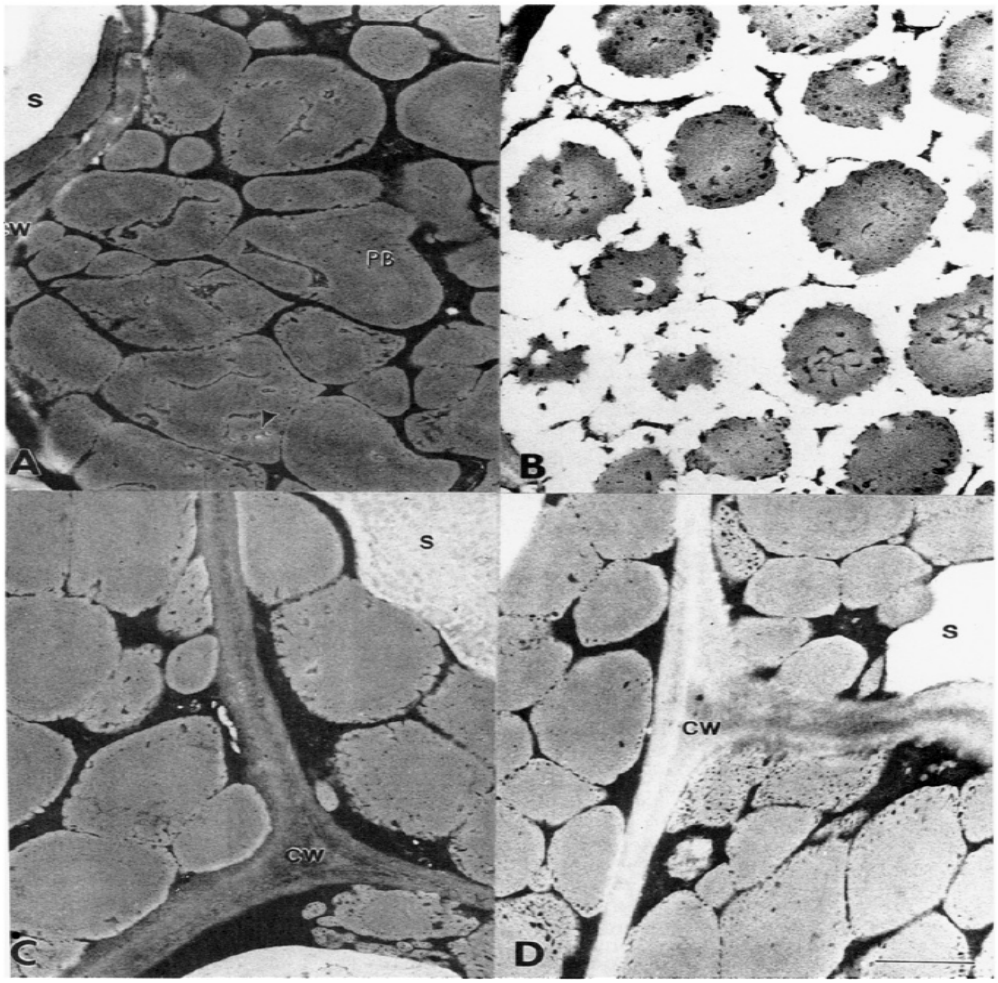


Figure 1.8 Electron micrographs of uncooked and cooked sorghum. (Oria et al, 1995).
(A) uncooked flour without pepsin digestion (arrow indicates protein body degradation); (B) uncooked flour after 120 min of pepsin digestion; (C) cooked flour without pepsin digestion; (D) cooked flour after 120 min of pepsin digestion. CW, cell wall; PB, protein body; S, starch. Bar =1μm.

micrographs to determine the influence of cooking (Fig. 1.8). The protein bodies were embedded in a dark staining protein matrix. The protein bodies had concentric rings, many dark inclusions, and dark staining projections that extended from the periphery of the protein body to the interior. There were granules remained in uncooked sorghum flour (Fig. 1.8 A), because amyloglucosidase and α-amylase only partially digested the starch granules. After digestion with

pepsin, most of the protein matrix was digested away, and the protein bodies were extensively pitted at their surfaces (Fig. 1.8 B). Many protein bodies were almost completely digested with only a core or central fragment remaining. Fig. 1.8 C indicated that protein bodies in the undigested sample of cooked sorghum looked similar to the uncooked ones. Protein bodies were embedded in a dark staining protein matrix with dark staining inclusions and projections. After pepsin digestion, however, the majority of the protein bodies were unaffected by pepsin and no pitting was observed (Fig. 1.8 D). The protein bodies in both undigested and digested samples appeared more fibrous than those in the uncooked samples.

OBJECTIVES

The objectives of this work were to study the digestion profile of starch in sorghum grains differing in endosperm hardness and flour particle size, to determine the difference in digestion of starch with and without pepsin treatment, and to analyze the enzymatic kinetics and structural changes after cooking of sorghum flours. Ultimately, we hope to understand how protein matrix affects digestion of starch in sorghum and develop methods to control the digestion of starch in sorghum flour.

CHAPTER 2 - MATERIALS AND METHODS

MATERIALS

SORGHUM FLOURS

Sorghum flours were generously given by Dr. Scott Bean in Grain Marketing Production Research Center (GMPRC), USDA-ARS, Manhattan, KS. They were grown at Nebraska in 2002. All of them contained normal starch and no tannin. The three sorghums had hardness values of 93.4, 75.1 and 61.4, respectively, as measured by the single kernel characterization system (SKCS) (Bean et al 2006). Both the hard sorghum and soft sorghum were red grain samples while the median one was a white grain sample. The hard sorghum had an average 25.4 mg of kernel weight and 1.6 mm of kernel diameter, median sorghum 27.3 mg and 2.1mm, and the soft sorghum, 28.9 mg and 1.7 mm. Each sorghum was milled on a Udy mill with either a 0.25, 0.5, or 1.0 mm screen. Based on their hardness and particle size, these 9 sorghum samples were abbreviated as follows: HS – hard and small, HM – hard and median, HL – hard and large, MS – median and small, MM – median and median, ML – median and large, SS – soft and small, SM – soft and median, and SL – soft and large. The information of the samples is shown in Table 2.1.

COOKED FLOURS

To prepare cooked samples, sorghum flour (~0.6 g) was weighed and placed in a 45 mL tube. Water was added to make 50% water content. Samples were cooked in a water bath at 90°C for 30 min. The cooked samples were cooled to room temperature. Before digestion, the cooked samples were homogenized by hand using a spatula.

CHEMICALS AND REAGENT

Fluorescamine (catalog # F2332) was purchased from Invitrogen Co., Eugene, OR;
Ethanol (catalog # E200, 111000200, 111ACS200, 111USP200) was purchased from Pharmco Inc., Brookfield, CT;
5,5'-dithiobis 2-nitrobenzoic acid (DTNB) (catalog # 117540050) was purchased from Acros Organics, Geel, BELGIUM;

Table 2.1 Hardness, particle size, protein content, total starch and moisture content of 9 sorghum flour samples

Sample	Grain Hardness Value	Flour Particle Size (μm)	Protein Content (%)	Total Starch (%)	Moisture Content (%)
HS	93.4	87.88	9.22±0.01	88.46±2.74	9.11
HM		227.94			10.84
HL		305.90			11.00
MS	75.1	88.48	9.19±0.01	81.12±2.51	9.48
MM		197.94			10.77
ML		311.65			11.42
SS	61.4	89.43	8.99±0.05	86.18±1.82	10.20
SM		190.94			10.75
SL		298.08			11.41

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO):

Pepsin, catalog # P-7000;

Pancreatin, catalog # P-7545;

Gum Guar, catalog # G-4129;

D-(+)-Glucose, catalog # G-7528;

Amyloglucosidase, catalog # A-7255;

α-Amylase, catalog # A-3176;

Ethylenediamine Tetraacetic Acid (EDTA), catalog # E-5134;

Sodium Dodecyl Sulfate (SDS), catalog # L-5750.

The following chemicals were purchased from Thermo Fisher Scientific Inc. (Pittsburgh, PA):

Hydrochloric Acid (HCl), catalog # A144-212;

Potassium Hydroxide (KOH), catalog # P250-1;

Acetic Acid 1N Solution, catalog # SA36-1;

Acetonitrile, catalog # A21-1;

Urea, catalog # U15-500;

Tris (Tromethamine), catalog # T393-500.
Sodium acetate trihydrate (NaAc) (catalog # 71188) and sodium tetraborate anhydrous (catalog # 71997) were purchased from Fluka Chemical Co. (Milwaukee, WI).
D-Glucose Assay Kit (catalog # K-GLUC) and Amylose/Amylopectin Assay Kit (catalog # K-AMYL) were purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland).

METHODS

ANALYSIS METHODS

Particle size was determined by Laser Diffraction Sizing (LDS) using a Beckman/Coulter LS 13 320 Laser Diffraction Particle Size Analyzer (Beckman/Coulter Particle Characterization, Miami, FL) (Wilson et al, 2006). Protein was determined by nitrogen combustion using a nitrogen determinator (Leco FP-528, St. Joseph, MI), according to AACC Approved Method 46-30 (AACC 2000). Nitrogen values were converted to protein content by multiplying by 6.25. Total starch (TS) was determined by glucose determination after dissolving in KOH solution (Englyst et al 1992). The moisture content was determined by using AACC Approved Method 44-15A (AACC 2000).

PEPSIN TREATMENT, ISOLATION OF STARCH FROM SORGHUM FLOUR, AND PROTEIN DIGESTIBILITY

The pepsin treatment initially was performed based on the procedure of Englyst starch kit. Sorghum (~0.6 g) was weighed and placed into a 45 mL tube with 50.0 mg guar gum. Pepsin solution was prepared by dissolving 50.0 mg pepsin in 10.0 mL 0.05M HCl. Pepsin solution (10 mL) was added into each sample and the mixtures were incubated in a water bath of 37°C for 30 min. We found that pH was 1.3 when pepsin (50 mg) was dissolved in 10.0 mL of 0.05M HCl solution. Pepsin preparation was modified by dissolving pepsin with 0.01M HCl (pH 2.0) instead of 0.05M HCl (pH 1.3) because pH 2.0 is the optimum pH for pepsin (Schlamowitz and Peterson, 1959). Incubation time was extended to 2 hr., 3 hr., and 4 hr. to determine the differences of starch and protein digestibility with different pepsin treatment time. Sodium acetate buffer (10.0 mL of 0.25M) was added to terminate pepsin treatment. The treatment was also terminated at 15 min., 30min., and 1hr., of incubation. All the samples with 6 different pepsin-treatment times (15min., 30min., 1hr., 2hr., 3hr., and 4hr.) were centrifuged at 1000*g* for 10 min. The supernatant was kept to determine nitrogen values for protein digestibility. The residues were freeze-dried and used for further analysis, such as protein digestibility determination, starch digestion, HPLC analysis, and confocal micrographs. The sample with 4-hr pepsin treatment was used as isolated starch because there was less than 0.5% of protein left.

STARCH DIGESTION TEST

Starch digestibility was determined by a modified Englyst method (Englyst et al 1992) to measure total starch and different starch fractions - RDS, SDS and RS, in each sample (~0.6 g) with and without pepsin digestion prior to the starch digestion. Glass beads (~8.4 g) were added into the mixtures for digestion. At the same time, the control was prepared with 50.0 mg gum in 20.0 mL 0.1M NaAc buffer (pH 5.2) and standard was prepared with 50.0 mg guar gum in 20.0 mL glucose standard solution (1.25 g glucose in 50.0 mL 0.1M NaAc buffer). To all the samples, control and standard were added 5.0 mL enzyme solution, and the tubes were shaken in a water bath of 37°C at 90 stroke/min. At 20 and 120 min interval, 250 μL of mixture was taken and transfered into 10.0 mL 66.6% ethanol solution, mixed well immediately and centrifuged at 1000*g* for 5 min. Supernatant (100 μL) was taken for colorimetric determination of glucose concentration at 510 nm by a D-glucose assay kit from Megazyme (Wicklow, Ireland). RDS (dry basis) content was calculated with the glucose concentration of 20 min interval (D20) by the equation of "0.9×D20× (25+ weights)/dry weight/1000×100". The glucose concentration of 120 min interval (D120) was applied into the same equation "0.9×D120× (25+weight)/dry weight/1000×100" to obtain digestible starch. Digestible starch measures RDS and SDS. SDS was estimated by the difference between digestible starch and RDS. RS was calculated by the difference between total starch and digestible starch.

AMYLOSE CONTENT DETERMINATION

Amylose content was determined by a Megazyme Amylose/Amylopection Assay Kit using a ConA method. Sorghum flour (~0.1 mg) was weighed into a 10 mL screw capped sample tube. DMSO 1 mL was added to the tube while gently stirring at low speed on a vortex mixer. The tube was capped and heated in a boiling water bath until the sample was completely dispersed. The contents of the sealed tube was vigorously mixed at high speed and then placed in a boiling water bath again and heated for 15 min., with intermittent high-speed stirring on a vortex mixer. 2 mL of 95% (v/v) ethanol was added with continuous stirring after the sample was stored at room temperature for approximately 5 min. A further 4 mL of ethanol was added and tube was capped to invert for mixing. The sample was placed at room temperature for 15 min to precipitate starch. The starch pellet was obtained by 5-min centrifugation at 2,000 g and draining away the ethanol. Two mL of DMSO was added to the starch pellet with gentle vortex

mixing and the sample was placed in a boiling water bath for 15 min with occasional mixing to ensure that there were no gelatinous lumps. Concanavalin A (Con A) solvent (4 mL) was immediately added with thorough mixing upon removing the tube from the boiling water bath. The contents were washed repeatedly with Con A solvent to a 25 mL volumetric flask. The quantitatively transferred contents were diluted to volume with Con A solvent. Sample was transferred to a 2.0 mL Eppendorf® microfuge tube with 1 mL and to another 10 mL tube with 0.5 mL. Sodium acetate buffer (4 mL, 100 mM, pH 4.5) and 0.1 mL of amyloglucosidae/α-amylase enzyme solution were added into the 10 mL tube and the mixture was incubated at 40°C for 10 min. This solution was transferred to a glass test tube with 1.0 mL and 4 mL GOPOD reagent was added with mixing. The absorbance at 510 nm was measured after incubation at 40°C for 20 min. and recorded as Total Starch Absorbance.

Con A solution (0.50 mL) was added into the Eppendorf® tube. The tube was capped and gently mixed by repeated inversion. The sample was centrifuged at 14,000 g for 10 min after 1 hr incubation at room temperature. The supernatant (1 mL) was transferred to a 15 mL centrifuge tube and 3 mL of 100 mM sodium acetate buffer (pH 4.5) was added. The sample was mixed and heated in a boiling water bath for 5 min. to denature the Con A. The sample was transferred into a water bath at 40°C to equilibrate for 5 min. 0.1 mL of amyloglucosidae/α-amylase enzyme mixture was added and the sample was incubated at 40°C for 30 min. After incubation, the tube was centrifuged at 2,000 g for 5 min. The supernatant (1.0 mL) was taken into another tube and 4 mL of GOPOD reagent was added. The sample was incubated at 40°C for 20 min. and measured absorbance at 510 nm recorded as Amylose Absorbance.

Amylose content was calculated as follows,

$$\text{Amylose, \% (w/w)} = \frac{\text{Amylose Absorbance}}{\text{Total Starch Absorbance}} \times \frac{6.15}{9.2} \times \frac{100}{1}$$

$$= \frac{\text{Amylose Absorbanc}}{\text{Total Starch Absorbance}} \times 66.8$$

Where 6.15 and 9.2 are dilution factors for the Amylose and Total Starch extracts respectively.

HPLC ANALYSIS

Kafirins were extracted from 100 mg of sample with 1 mL of 60% tertiary butanol containing 0.5% sodium acetate and 2% beta mercaptoethanol (Ioerger et al, 2007). Samples were heated at 80°C for 2 min to deactivate any enzymatic activity in the samples. Proteins were

analyzed using a Poroshell C8 column (5 μL injection) on an Agilent 1100 HPLC system with solvent A being 0.1% triflouracetic acid (TFA) (w/v) in water and solvent B acetonitrile plus 0.07% TFA (w/v). The separation was achieved using a linear gradient of solvent B from 45 to 60% over 19 min. Column temperature was maintained at 50°C with a flow rate of 0.7 mL/min. Kafirin subclasses in the chromatograms were identified by comparing results to that of Bean et al 2000.

CONFOCAL MICROSCOPY

Proteins were labeled using a fluorescamine dye according to the method of Bantan-Polak et al (2001). Fluorescamine dye (300 μL, 0.1% (w/v)) was dissolved in acetonitrile followed by 50 μL of 0.1 M sodium tetraborate buffer, pH 8.0, and 150 μL of water. Approximately 50 mg of sample was weighed and added into the above solution. The samples were stained for 20 min and rinsed off with deionized water.

The sample was observed using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss, Munich, Germany). Excitation of fluorescamine-labeled proteins was at 360 nm. 450/80 band pass filter was used to detect fluorescamine. Digital images were processed using Leica CM3050S Cryostat software (Carl Zeiss, Munich, Germany).

DETERMINATION OF SULFHYDRYL GROUPS

Free sulfhydryl group content was determined using the direct colorimetric method of Chan and Wasserman (1993). The experiment was conducted under dark conditions. A sample size of 30 mg in 2.5-mL Eppendorf® tubes was used. The reaction buffer consisted of 1 mL of 8M urea, 3 mM EDTA, 1% SDS, 0.4 mM 5,5'-dithiobis 2–nitrobenzoic acid (DTNB) (Sigma), and 0.2M Tris-HCl pH 8.0 buffer. The samples were incubated at room temperature (~20°C) for 30 min, with vortexing every 10 min After 30 min, samples were centrifuged at 7,200 × g for 15 min, and the absorbance of the clear supernatant read at 412 nm after dilution with 0.2M Tris-HCl buffer pH 8.0 containing 8M urea, 3 mM EDTA, and 1% SDS. Experiments for determination of sulfhydryl group contents were conducted in triplicate.

STATISTICAL ANALYSIS

SAS (SAS Institute Inc., Cary, NC) was used to conduct two-way analysis of variance (ANOVA) for determining significant differences among means of starch digestion values.

Statistically significant differences ($P<0.05$) among means were determined using a Tukey multiple comparison procedure.

CHAPTER 3 - RESULTS AND DISCUSSION

EFFECT OF PH ON PROTEIN AND STARCH DIGESTIBILITY

In the pepsin treatment experiments, when pH was 1.3, 6 to 20% protein was digested (Table 3.1 and Fig. 3.1). In contrast, when pH of 2.0 was used, 42 to 64% protein digested. These results suggest it is important to have pH of 2.0 during the pepsin treatment to ensure that large amount of protein is hydrolyzed.

At pH 2.0, starch digestibility after the pepsin treatment increased (Table 3.2 and Fig. 3.2), suggesting that the protein matrix affects the starch digestion.

Table 3.1 Effect of pH on protein digestion of 9 native sorghum flour samples

Sample	Protein Digestibility (%)	
	pH 1.3	pH 2.0
HS	19.23±0.05	64.19±0.18
HM	15.10±0.12	58.99±0.09
HL	9.41±0.01	55.03±0.21
MS	16.47±0.01	53.43±0.16
MM	12.45±0.02	47.62±0.19
ML	6.85±0.06	42.30±0.06
SS	16.97±0.09	57.41±0.01
SM	13.58±0.11	46.05±0.17
SL	7.87±0.06	44.98±0.20

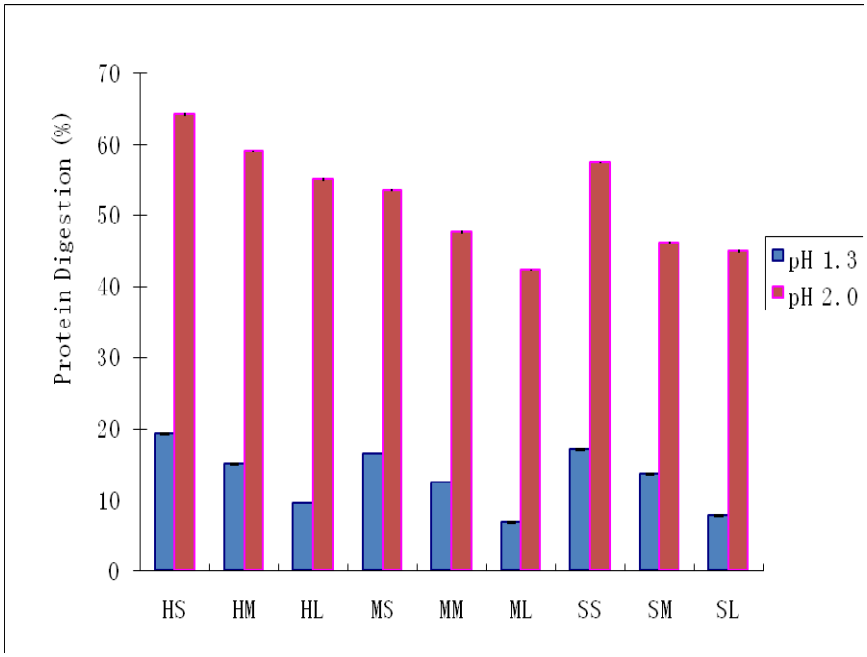


Figure 3.1 Effect of pH on protein digestion of 9 native sorghum flour samples.

Table 3.2 Effect of pH on starch digestion of 9 native sorghum flour samples*

Sample	pH 1.3			pH 2.0		
	RDS (%)	SDS (%)	RS (%)	RDS (%)	SDS (%)	RS (%)
HS	37.19±0.47^{a}	44.33±1.13^{a}	18.48±0.75^{a}	37.74±0.36^{a}	51.65±0.74^{a}	10.61±0.55^{a}
HM	22.66±1.26^{b}	51.77±0.74^{b}	25.57±1.00^{b}	22.99±1.04^{b}	58.92±0.64^{b}	18.09±0.84^{b}
HL	19.60±0.64^{c}	49.53±1.22^{c}	30.87±0.93^{c}	20.13±0.27^{c}	57.29±0.37^{c}	22.58±0.32^{c}
MS	34.44±1.05^{d}	45.55±1.05^{d}	20.01±1.05^{d}	33.78±0.07^{d}	51.25±0.19^{a}	14.97±0.13^{d}
MM	24.26±0.04^{b}	46.26±0.96^{d}	29.48±0.50^{c}	23.59±0.74^{b}	53.59±0.99^{d}	22.82±0.86^{c}
ML	16.26±0.46^{f}	50.16±1.29^{b}	33.60±0.87^{e}	17.90±1.16^{e}	52.56±0.52^{d}	29.54±0.83^{c}
SS	30.30±1.06^{g}	51.55±0.19^{b}	18.15±0.55^{a}	31.28±0.92^{f}	57.96±1.77^{c}	10.76±0.84^{a}
SM	30.93±0.86^{g}	44.39±0.22^{e}	24.68±0.53^{b}	28.91±1.24^{g}	51.47±0.35^{a}	19.62±0.79^{b}
SL	18.02±0.08^{c}	53.44±0.45^{f}	28.54±0.26^{f}	18.68±0.49^{e}	56.70±0.09^{c}	24.62±0.29^{e}

*Values are means±SD. Means not sharing a common superscript letter in a column are significantly different at $p \leq 0.05$.

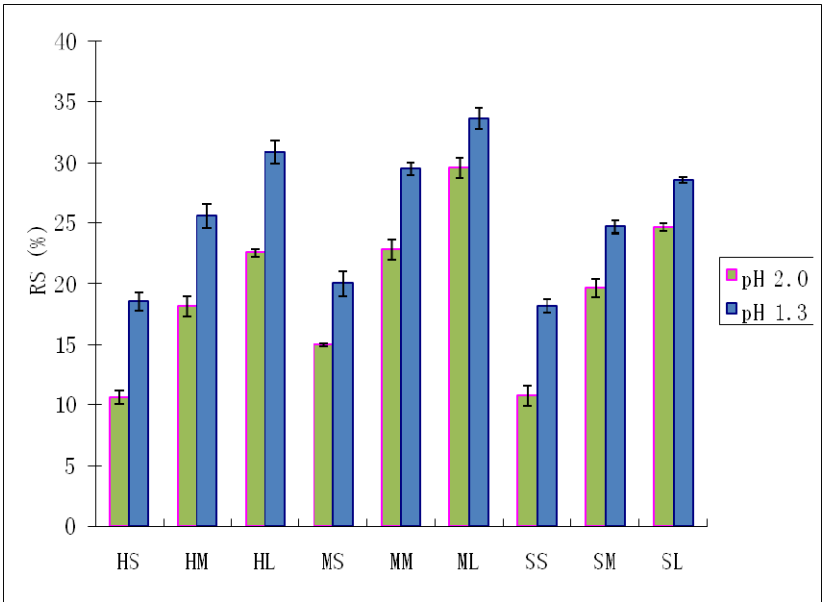


Figure 3.2 Effect of pH on starch digestion of 9 native sorghum flour samples (RS content).

EFFECT OF PARTICAL SIZE AND HARDNESS ON DIGESTION

Flour from the hard sorghum gave higher protein and starch digestibility than flour from median and soft ones. But there was no significant difference between median and soft flours (Table 3.3 and Fig. 3.3-3.5).

Starch digestibility increased with the pepsin treatment (Fig. 3.3-3.5). RDS content was similar with or without the pepsin treatment (Fig. 3.4). The differences were almost less than 1%. RS content had large difference when pepsin treatment was applied (Fig. 3.3), ranging from 3.39% to 9.14%. The sorghum flour with median hardness and small particle size had 24.11% of RS whereas RS was only 14.97% after pepsin treatment, indicating the starch digestibility increased after pepsin treatment. Compared with hard and soft sorghum flours, the median one had relatively higher RS content, which was 24.1-35.5%, compared to 19.0-28.4% in hard and soft flours.

In agreement with previous work (Owsley, et al, 1981), decreasing particle size increased starch digestion (Fig. 3.3-3.5).

Early studies has illustrated that decreasing sorghum particle size improves both starch and protein digestibility (Luce et al, 1970 and Lawrence, 1970). As a consequence, successive reductions in sorghum particle size increase nutritional value (Owsley et al, 1981).

Hardness or corneousness in sorghum and corn is related to protein content and continuity of the protein matrix (Rooney and Miller, 1982). The matrix may be continuous or incomplete and consists of glutelins in which starch granules and prolamine-rich protein bodies are embedded. In corneous endosperm, starch granules are smaller and the matrix nearly continuous. Floury endosperm cells tend to have more and larger starch granules surrounded by a discontinuous matrix with fewer protein bodies. The resistance to digestive action of the hard peripheral endosperm layer is largely responsible for low digestibility. In my results, the starch digestibility was lower in median sorghum than in hard and soft one. There must be another factor such as amylose content affecting more than hardness. It should be noted that the median sorghum was white grain, whereas the others were red grain.

Table 3.3 Starch digestion of 9 native sorghum flour samples with different particle sizes and hardness*

Sample	RDS (%)		SDS (%)		RS (%)	
	w/ pepsin	w./o. pepsin	w/ pepsin	w./o. pepsin	w/ pepsin	w./o. pepsin
HS	37.74±0.36^{a}	36.51±0.26^{a}	51.65±0.74^{a}	44.46±1.02^{a}	10.61±0.55^{a}	19.03±0.64^{a}
HM	22.99±1.04^{b}	22.47±0.29^{b}	58.92±0.64^{b}	51.27±0.40^{b}	18.09±0.84^{b}	26.26±0.35^{b}
HL	20.13±0.27^{c}	19.30±1.25^{c}	57.29±0.37^{c}	52.35±0.97^{b}	22.58±0.32^{c}	28.35±1.11^{c}
MS	33.78±0.07^{d}	32.85±0.42^{d}	51.25±0.19^{a}	43.04±0.87^{a}	14.97±0.13^{d}	24.11±0.64^{d}
MM	23.59±0.74^{b}	23.40±0.46^{b}	53.59±0.99^{a}	46.01±0.66^{c}	22.82±0.86^{c}	30.59±0.55^{e}
ML	17.90±1.16^{e}	16.85±0.09^{e}	52.56±0.52^{a}	47.64±1.28^{c}	29.54±0.83^{e}	35.51±0.68^{f}
SS	31.28±0.92^{d}	30.49±1.54^{d}	57.96±1.77^{c}	49.98±0.04^{d}	10.76±0.84^{a}	19.53±0.89^{a}
SM	28.91±1.24^{d}	28.27±0.12^{d}	51.47±0.35^{a}	45.44±0.59^{c}	19.62±0.79^{b}	26.29±0.36^{b}
SL	18.68±0.49^{e}	18.18±1.00^{e}	56.70±0.09^{c}	53.81±1.31^{b}	24.62±0.29^{f}	28.01±1.16^{c}

* Values are means±SD. Means not sharing a common superscript letter in a column are significantly different at $p \leq 0.05$. w/ pepsin = with pepsin treatment; w./o. pepsin = without pepsin treatment.

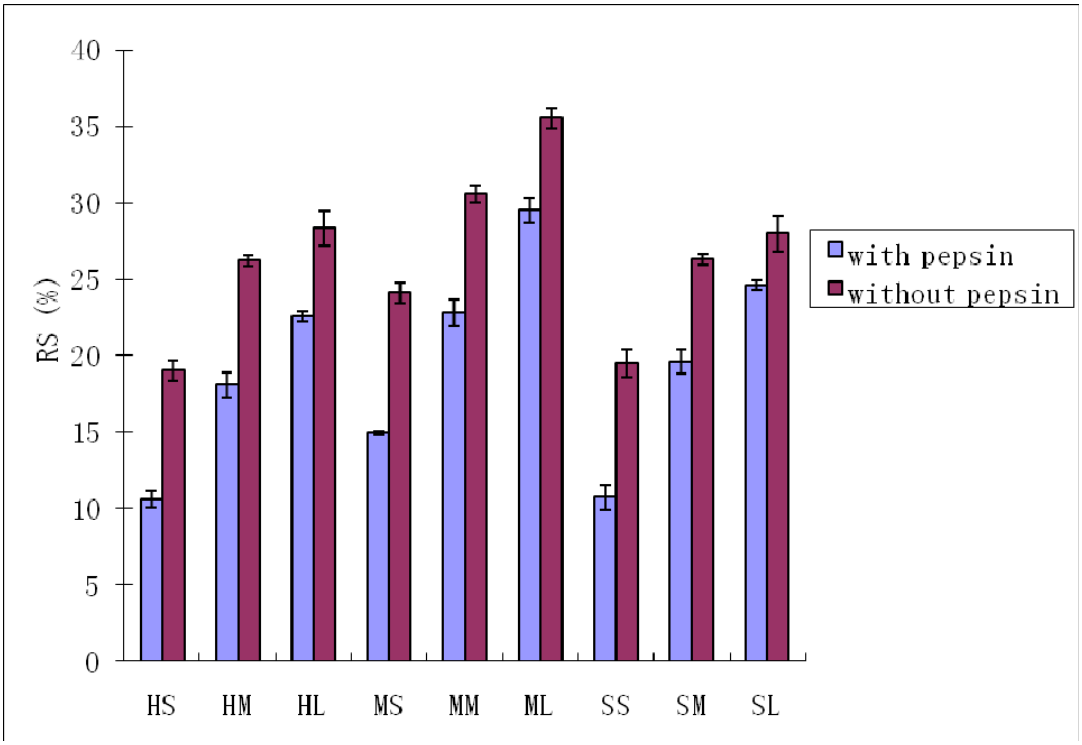


Figure 3.3 Comparison of RS content with and without pepsin treatment (pH 2.0) of 9 native sorghum flour samples with different particle sizes and hardness.

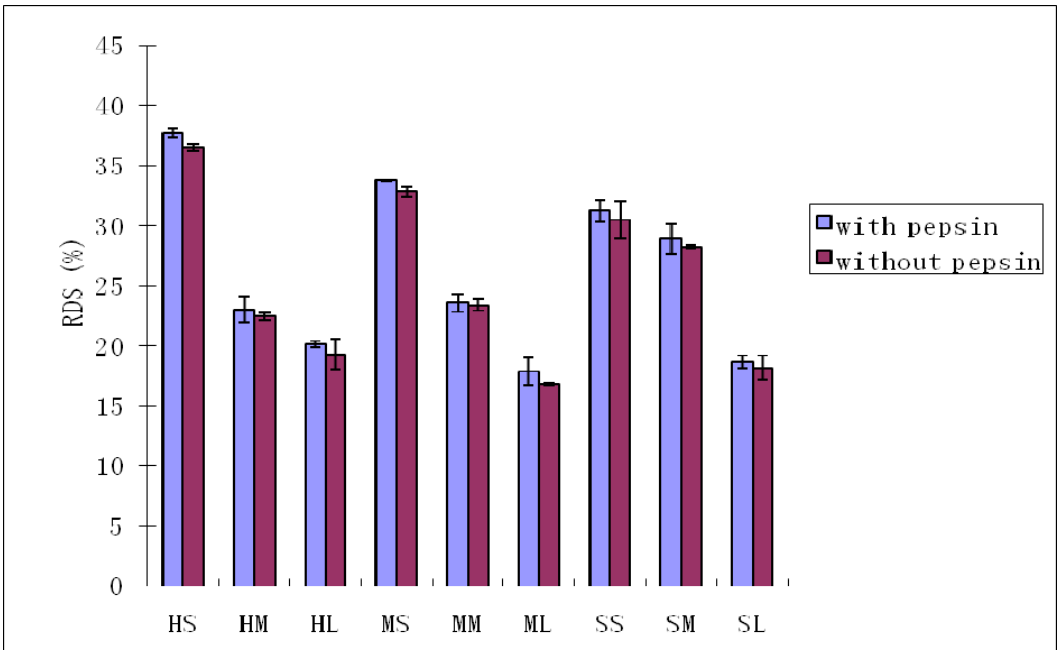


Figure 3.4 Comparison of RDS content with and without pepsin treatment (pH 2.0) of 9 native sorghum flour samples with different particle sizes and hardness.

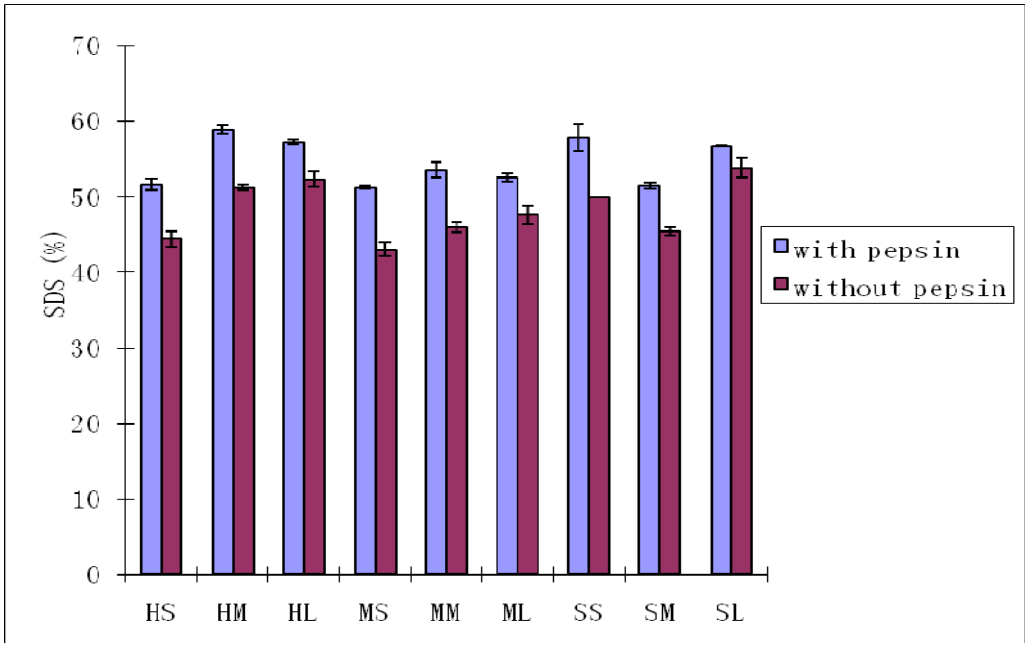


Figure 3.5 Comparison of SDS content with and without pepsin treatment of 9 native sorghum flour samples with different particle sizes and hardness.

EFFECT OF COOKING ON STARCH DIGESTION

Cooking affected both protein and starch digestion, decreasing protein digestion (Table 3.5) but increasing starch digestion (Table 3.4 and Fig. 3.6-3.8). RDS content increased (Fig. 3.6 and 3.9) when the samples were cooked, while SDS and RS content decreased (Fig. 3.7-3.8 and 3.10-3.11), with or without pepsin treatment. Similarly to native sorghum flours before cooking, there were no significant difference in RDS content after cooking when pepsin treatment was applied (Fig. 3.4 and 3.12). But the differences of RS and SDS content between with and without pepsin treatment increased when the flours were cooked (Fig. 3.13-3.14). The differences in RS content ranged from 10.5-15.5% after cooking. In comparison, before cooking, the range was 3.4-9.1%. As observed before cooking, the median sorghum flour had relatively higher RS content than hard and soft ones after cooking.

Table 3.4 Starch digestion of 9 cooked sorghum flour samples with different particle sizes and hardness*

Sample	RDS (%)		SDS (%)		RS (%)	
	w/ pepsin	w./o. pepsin	w/ pepsin	w./o. pepsin	w/ pepsin	w./o. pepsin
HS	56.01±0.39^{a}	54.78±1.14^{a}	39.13±0.87^{a}	28.29±0.14^{a}	4.86±0.63^{a}	16.93±0.64^{a}
HM	53.35±0.75^{b}	52.05±1.08^{a}	40.93±0.69^{a}	30.65±1.22^{a}	5.72±0.72^{a}	17.30±1.15^{a}
HL	52.17±0.84^{b}	51.46±0.05^{a}	38.59±0.55^{a}	27.96±0.51^{a}	9.24±0.69^{b}	20.58±0.28^{b}
MS	50.86±1.34^{c}	48.94±0.61^{b}	43.92±1.37^{b}	30.35±0.42^{a}	5.22±1.35^{a}	20.71±0.52^{b}
MM	46.97±0.43^{d}	45.55±0.79^{c}	42.76±0.84^{b}	30.92±0.16^{a}	10.27±0.64^{b}	23.53±0.48^{c}
ML	41.99±0.88^{e}	41.03±0.21^{d}	45.48±0.19^{b}	34.98±0.76^{b}	12.53±0.53^{c}	23.99±0.49^{c}
SS	49.11±0.61^{c}	47.43±0.46^{b}	42.88±0.48^{b}	33.23±0.17^{b}	8.01±0.55^{b}	19.34±0.33^{b}
SM	46.94±1.13^{d}	45.63±0.94^{b}	43.37±0.22^{b}	34.15±0.59^{b}	9.69±0.67^{b}	20.22±0.77^{b}
SL	44.13±0.91^{e}	43.55±0.84^{b}	46.67±0.54^{b}	36.18±0.38^{c}	9.20±0.73^{b}	20.27±0.61^{b}

* Values are means±SD. Means not sharing a common superscript letter in a column are significantly different at $p \leq 0.05$. w/ pepsin = with pepsin treatment; w./o. pepsin = without pepsin treatment.

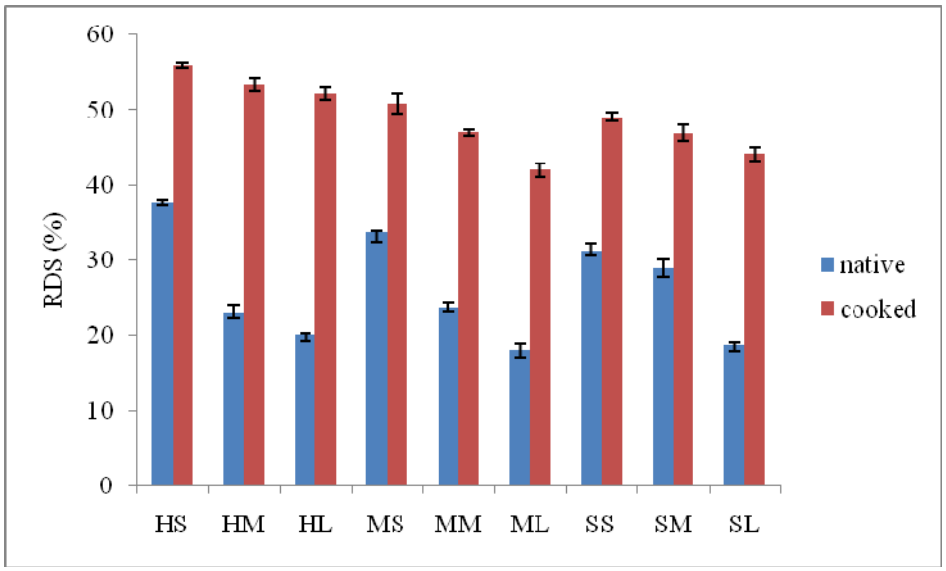


Figure 3.6 Comparison of RDS content before and after cooking with pepsin treatment of 9 sorghum flour samples with different particle sizes and hardness.

The particle size had little effect on starch digestion after cooking (Table 3.4), comparing the effect in native sorghum flours. There was no difference of starch digestibilities with different particle sizes of sorghum flours after cooking. Because the starches in sorghum flours were gelatinized when cooked and had to be homogenized before starch digestion, the particle sizes for cooked samples were no longer the original particle sizes of sorghum flours.

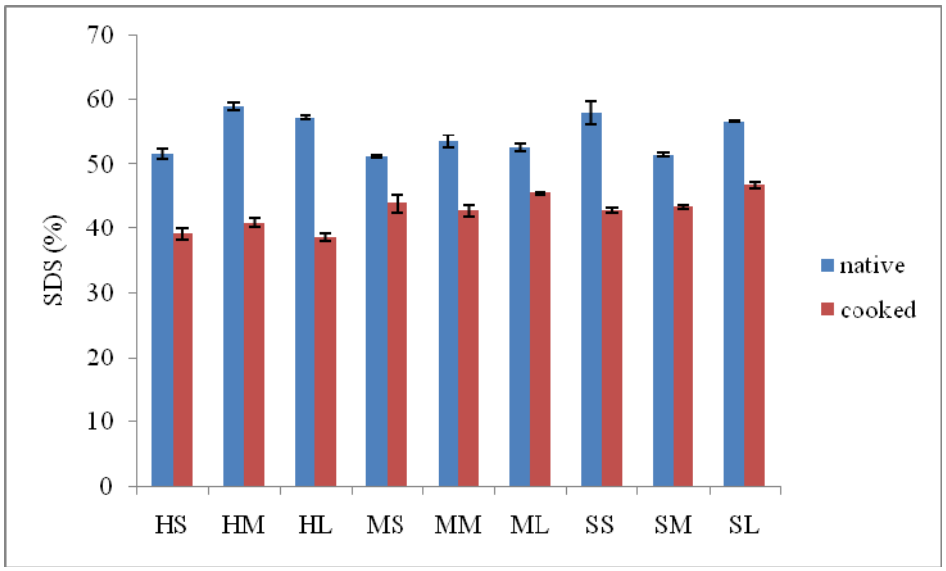


Figure 3.7 Comparison of SDS content before and after cooking with pepsin treatment of 9 sorghum flour samples with different particle sizes and hardness.

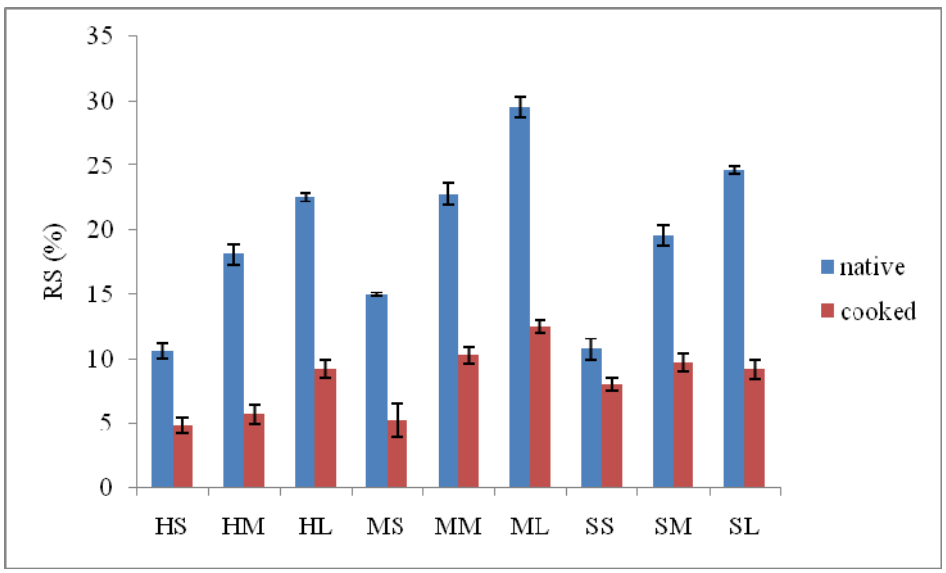


Figure 3.8 Comparison of RS content before and after cooking with pepsin treatment treatment of 9 sorghum flour samples with different particle sizes and hardness.

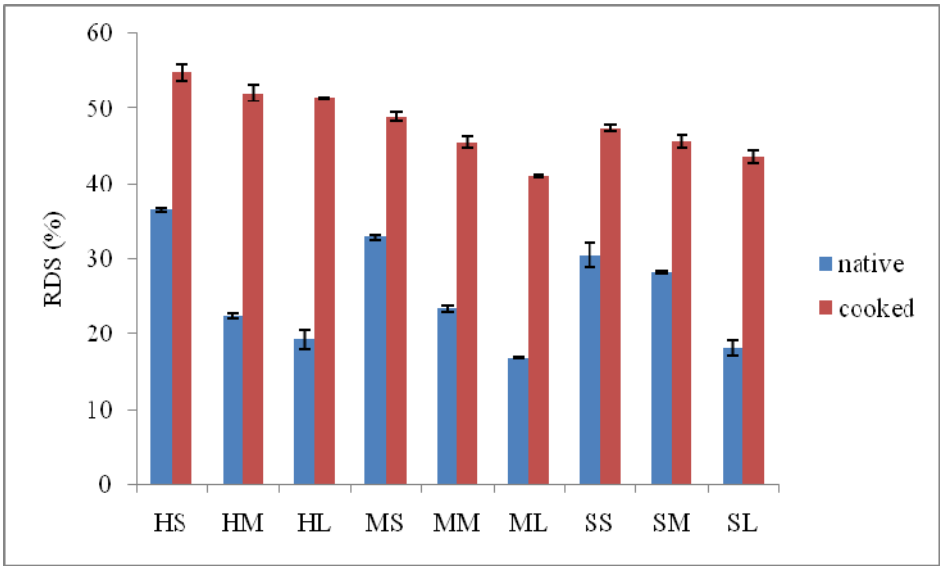


Figure 3.9 Comparison of RDS content before and after cooking without pepsin treatment of 9 sorghum flour samples with different particle sizes and hardness.

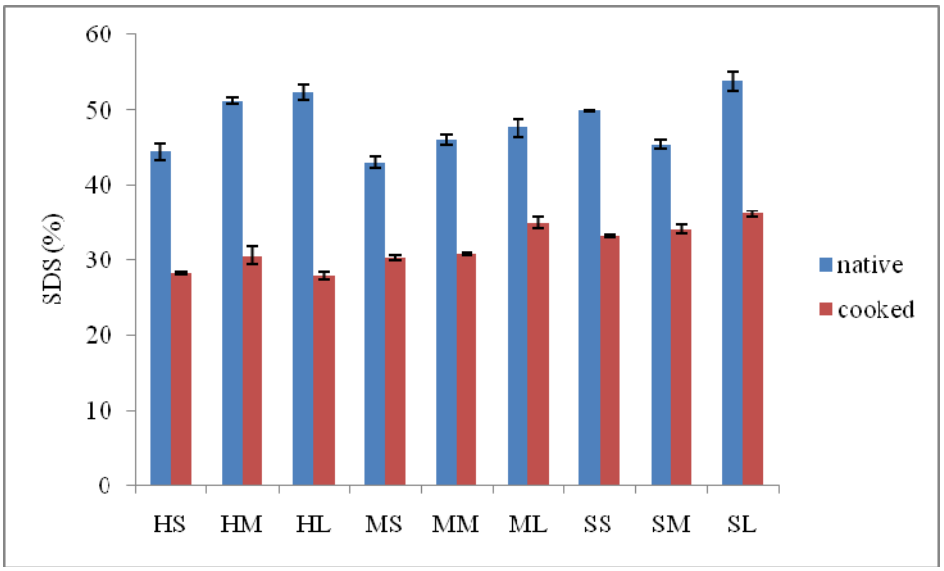


Figure 3.10 Comparison of SDS content before and after cooking without pepsin treatment of 9 sorghum flour samples with different particle sizes and hardness.

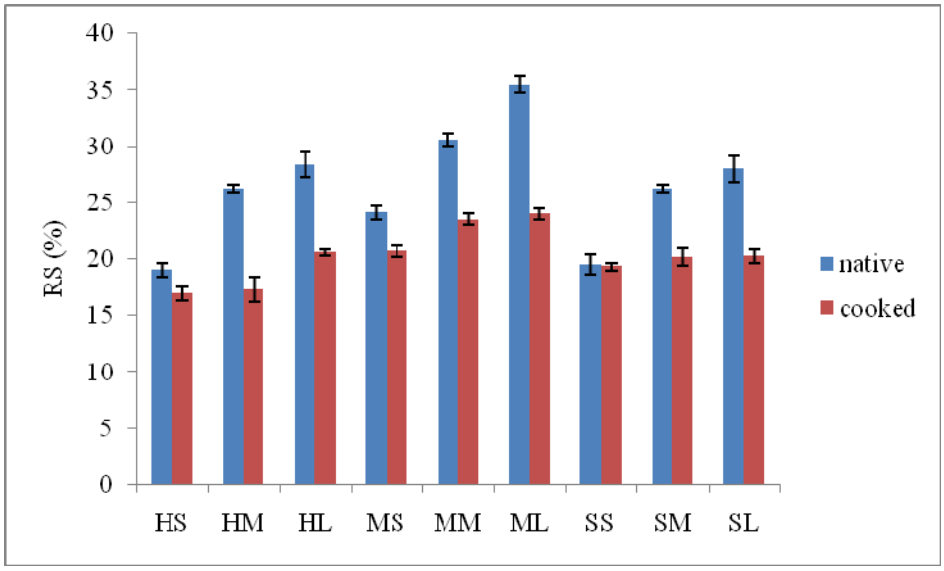


Figure 3.11 Comparison of SDS content before and after cooking without pepsin treatment of 9 sorghum flour samples with different particle sizes and hardness.

Figure 3.12 Comparison of RDS content with and without pepsin treatment of 9 cooked sorghum flour samples with different particle sizes and hardness.

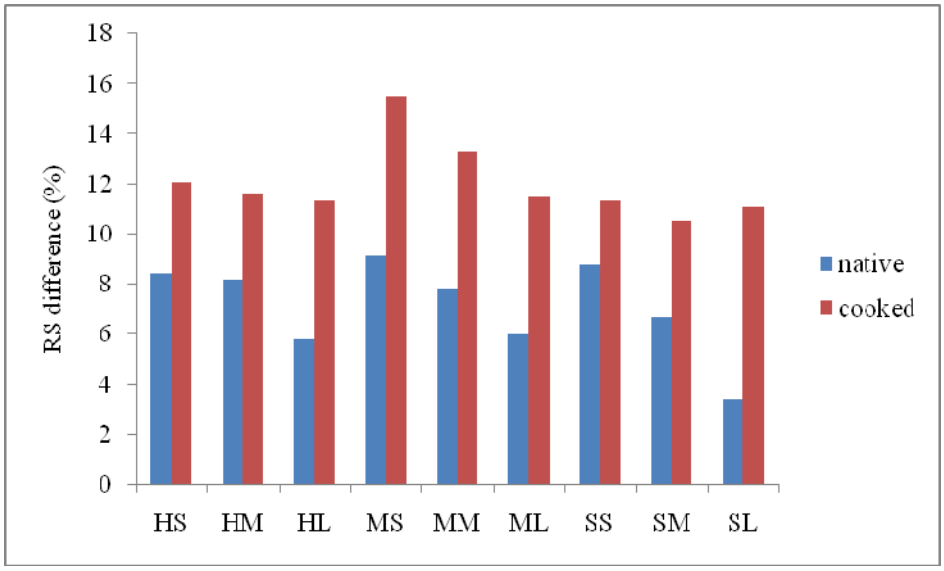


Figure 3.13 Comparison of RS difference of with and without pepsin treatment between native and cooked sorghum flours.

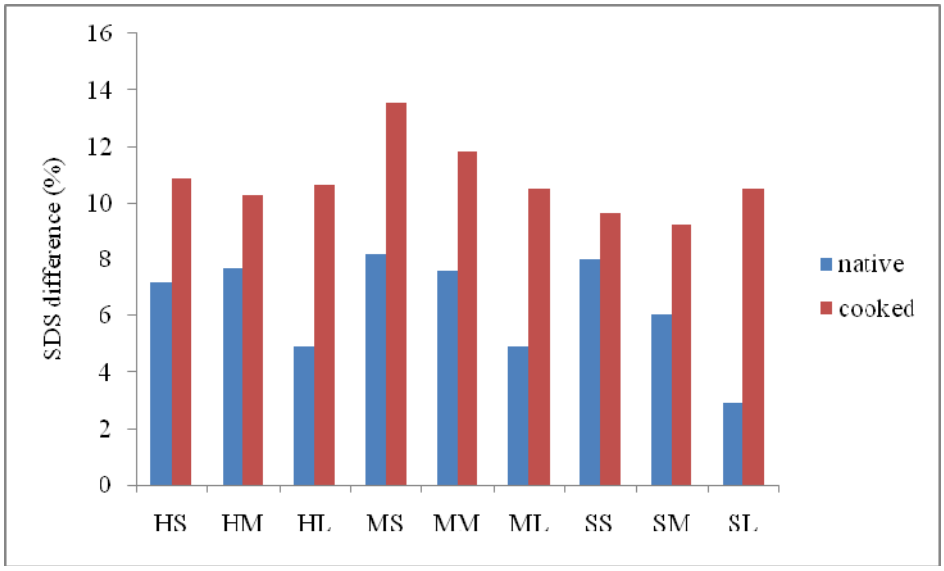


Figure 3.14 Comparison of SDS difference of with and without pepsin treatment between native and cooked sorghum flours.

EFFECT OF COOKING ON PROTEIN DIGESTIBILITY

In the Englyst test, the pepsin treatment was 30 min., and the differences in protein digestion between cooked and native sorghum flours were small (Table 3.5 and Fig. 3.16). When

the digestion time extended from 30 min to 2 hr., digestion of protein in cooked sorghum was much lower than that of protein in native flours (Table 3.5 and Fig. 3.15).

Table 3.5 Protein digestibility of 9 sorghum flour samples before and after cooking*

Pepsin treatment	2 hr		30 min	
Sample	Native	Cooked	Native	Cooked
HS	64.93±0.95	47.28±1.17	64.19±0.18	58.76±0.11
HM	63.67±0.67	49.12±0.43	58.99±0.09	56.71±0.20
HL	63.08±0.41	48.34±0.95	55.03±0.21	52.00±0.18
MS	65.11±1.04	45.67±0.69	53.43±0.16	54.30±0.09
MM	63.93±0.84	44.39±0.18	47.62±0.19	45.65±0.10
ML	64.27±1.99	44.37±1.33	42.30±0.06	43.49±0.06
SS	58.26±0.64	39.22±0.47	57.41±0.01	55.84±0.14
SM	58.03±0.35	41.78±0.68	46.05±0.17	48.37±0.29
SL	57.44±0.27	40.22±1.08	44.98±0.20	44.21±0.12

* Values are means±SD.

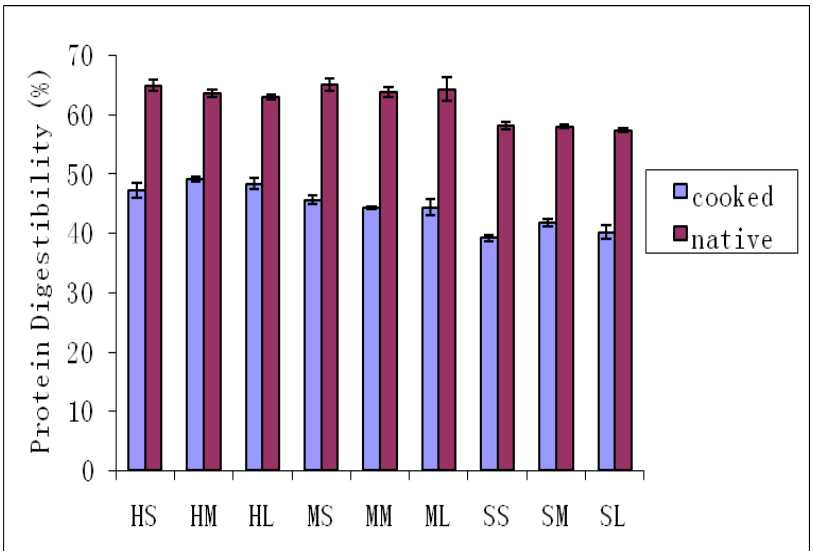


Figure 3.15 Protein digestibility of 9 sorghum flour samples before and after cooking (pepsin treatment: 37°C for 2 hr).

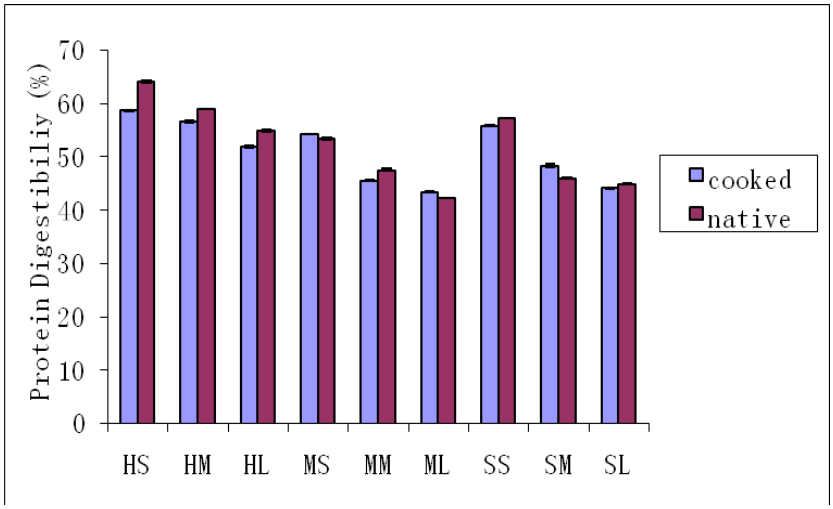


Figure 3.16 Protein digestibility of 9 sorghum flour samples before and after cooking (pepsin treatment: 37°C for 30 min.)

PROTEIN HYDROLYSIS

Data above (Table 3.5 and Fig. 3.15-3.16) indicated the possibility of incomplete protein digestion during pepsin treatment. We decided to extend treatment time to achieve complete protein digestion. Samples were treated by pepsin for 3 and 4 hours to compare with the results of 30 min and 2 hours (Fig. 3.17). After 4 hr pepsin treatment, protein was almost all hydrolyzed. The samples after 4-hr pepsin treatment were used as isolated starches. The protein content in isolated starches was around 0.5% (Table 3.6).

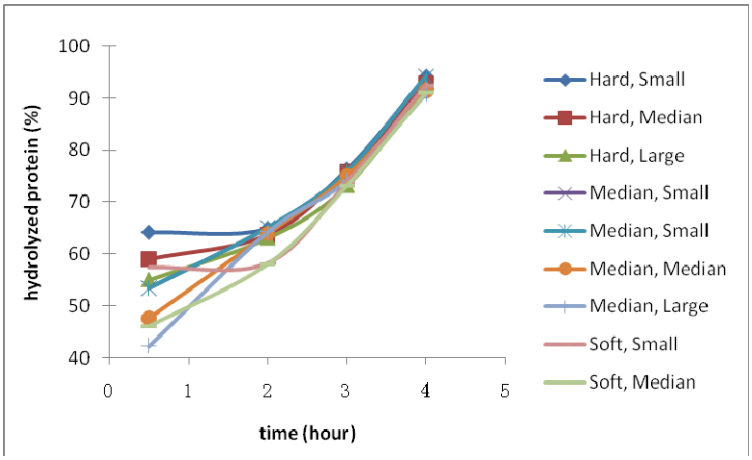


Figure 3.17 Hydrolysis of protein in 9 sorghum flour samples with time.

Table 3.6 Protein content in isolated starches from 9 sorghum flours.

Sample	Protein content (%)	Sample	Protein content (%)	Sample	Protein content (%)
HS	0.44	MS	0.55	SS	0.52
HM	0.53	MM	0.59	SM	0.48
HL	0.57	ML	0.57	SL	0.55

As a result of different protein digestibility at different pepsin treatment time, starch digestion was different, too (Fig. 3.18). The RS content decreased with increasing pepsin treatment time, but the decreasing rate was very slow. Most decrease occurred between 30 min to 2 hr. During 2-4 hr of pepsin treatment, only sorghum flour with median particle size had a decrease in RS content, while the sorghum flours with small and large particle size had no difference in RS content (Table 3.7-3.9). On the contrary, the rate of protein digestibility was slower between 30 min and 2 hr but faster from 2 hr to 4 hr. Since the sample after 4-hr pepsin treatment was considered as isolated starch, the starch digestibility of the isolated sorghum starch (8.47-26.28% of RS content). There may be Zhang et al (2000b) reported that normal maize starch had RS content of 22.6%. Actually, Englyst test needs to be standardized upon different starches.

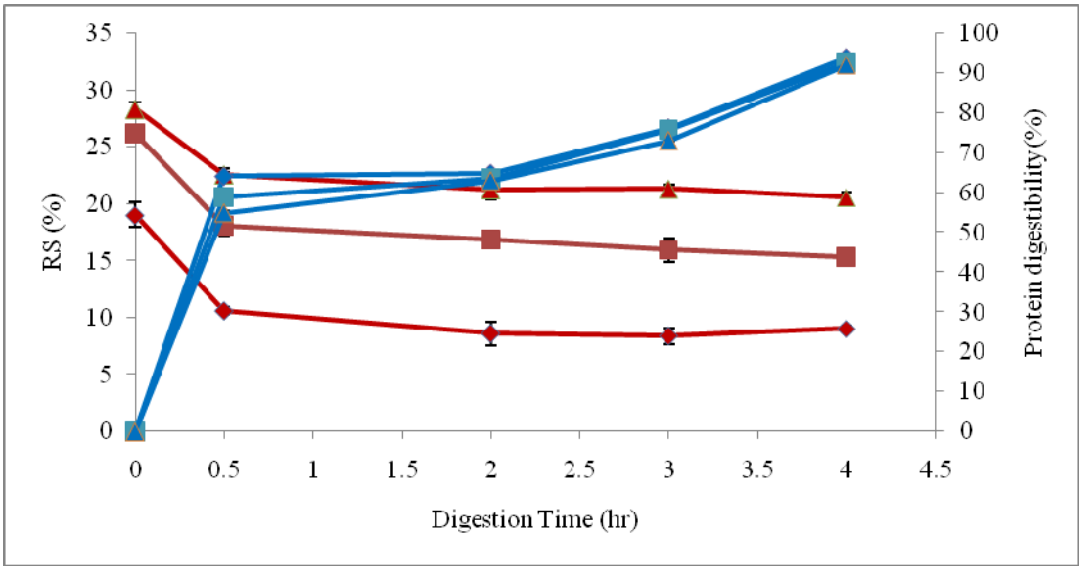


Figure 3.18 Resistant starch (RS) content and protein digestibility of hard sorghum flour with different particle sizes. red: RS content; blue: protein digestibility; ◆: small particle size; ■:median particle size; ▲: large particle size.

Table 3.7 Starch digestion of 9 native sorghum flour after 2 hr protein digestion*

Sample	RDS (%)	SDS (%)	RS (%)
HS	39.51±0.15^{a}	51.88±0.46^{a}	8.61±0.81^{a}
HM	25.04±0.94^{b}	58.11±0.94^{b}	16.85±0.05^{b}
HL	19.82±1.72^{c}	58.93±1.06^{b}	21.25±1.01^{c}
MS	37.15±1.37^{a}	51.96±0.85^{a}	10.89±1.21^{a}
MM	25.16±0.61^{b}	55.26±1.78^{c}	19.58±0.53^{d}
ML	20.74±0.21^{c}	51.93±0.45^{a}	27.33±0.78^{e}
SS	34.96±0.56^{d}	59.54±0.95^{b}	8.41±0.59^{a}
SM	28.14±0.95^{e}	53.56±0.17^{d}	18.30±1.09^{d}
SL	20.19±0.93^{c}	57.22±1.11^{b}	22.59±0.36^{c}

* Values are means±SD. Means not sharing a common superscript letter in a column are significantly different at $p \leq 0.05$.

Table 3.8 Starch digestion of 9 native sorghum flour after 3 hr protein digestion*

Sample	RDS (%)	SDS (%)	RS (%)
HS	38.28±0.21[a]	53.23±0.57[a]	8.39±0.43[a]
HM	26.01±0.33[b]	58.07±0.61[b]	15.92±1.05[b]
HL	20.53±0.85[c]	58.17±0.99[b]	21.30±0.73[c]
MS	37.02±0.52[a]	51.41±0.36[c]	11.57±0.72[a]
MM	25.89±0.63[b]	55.69±1.02[d]	18.42±0.56[d]
ML	20.00±0.89[c]	52.31±0.51[a]	27.69±1.06[e]
SS	36.36±0.26[a]	55.68±0.33[d]	6.96±0.18[a]
SM	27.83±0.97[b]	53.64±0.53[a]	18.53±0.17[d]
SL	20.84±0.69[c]	58.22±0.17[b]	20.94±1.32[c]

* Values are means±SD. Means not sharing a common superscript letter in a column are significantly different at $p \leq 0.05$.

Table 3.9 Starch digestion of 9 native sorghum flour after 4 hr protein digestion*

Sample	RDS (%)	SDS (%)	RS (%)
HS	39.15±0.77[a]	51.84±0.65[a]	9.01±0.43[a]
HM	26.88±0.28[b]	57.83±0.38[b]	15.29±0.18[b]
HL	19.47±0.93[c]	59.95±0.25[c]	20.58±0.31[c]
MS	37.92±0.36[a]	51.19±0.02[a]	10.89±1.51[a]
MM	26.13±0.48[b]	54.76±0.32[d]	19.11±0.49[d]
ML	19.69±0.69[c]	54.03±0.85[d]	26.28±0.99[e]
SS	37.82±1.09[a]	53.71±0.44[d]	8.47±0.41[a]
SM	27.35±0.22[b]	55.02±0.29[d]	17.63±0.92[d]
SL	19.96±0.53[c]	59.16±1.03[c]	20.88±0.05[c]

* Values are means±SD. Means not sharing a common superscript letter in a column are significantly different at $p \leq 0.05$.

AMYLOSE CONTENT

Amylose content in these samples is shown in Table 3.10.

Table 3.10 Amylose content in 9 sorghum samples with different particle sizes and hardness*

Sample	Amylose Content (%)
HS	20.1±0.39
HM	20.0±0.22
HL	20.2±0.14
MS	24.0±0.07
MM	23.8±0.41
ML	23.9±0.29
SS	19.8±0.37
SM	20.0±0.11
SL	20.1±0.23

* Values are means±SD.

The flour from median hardness sample had the highest amylose content. The higher amylose content in median sorghum flour probably contributed to the higher RS content. However, Zhang et al (2006a) reported that amylose did not have a significant impact on the slow digestion property of normal maize starch. Since the composition of starch is amylose and amylopectin, high amylose content indicates low amylopectin content. As discussed previously in the introduction, the organized structure of amylopectin is associated with enzyme accessibility to starch granules and finally affects starch digestion (Oates, 1997). Starch digestibility would be high in samples with low amylopectin content.

HPLC RESULTS

There were 3 areas of protein selected in HPLC chromatograph, representing γ-kafirin, α/β-kafirin, and α-kafirin. α-Kafirin, the major sorghum storage protein, makes up about 60-70% of the total protein in the grain (Aboubacar et al, 2001).

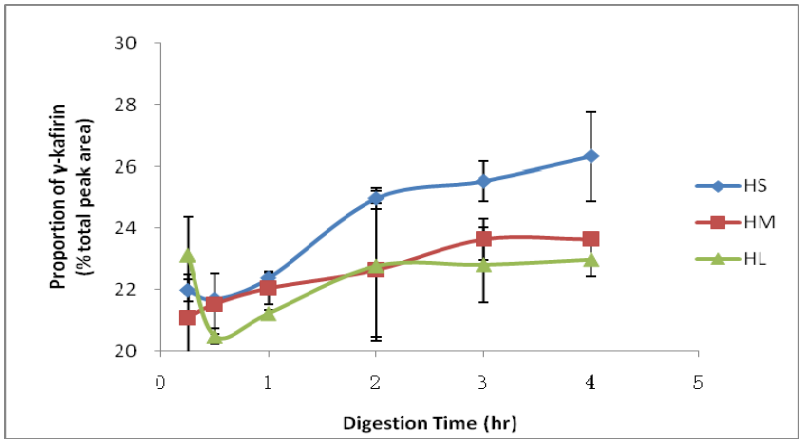
The percentage of the three areas is shown in Table 3.11. Compared with different particle sizes, γ-kafirin had a trend of increasing with pepsin treatment times (Fig. 3.19), while the other peaks showed decreased (Fig. 3.20-3.21), though there were several occasional exceptions. Compared with different hardness (Fig. 3.22-3.24), there was no significant trend for different proteins. As these samples were treated with pepsin prior to HPLC analysis, hydrolysis

of the proteins may have caused changes in elution times of some of the remaining proteins, thus interfering with quantification of the proteins.

Table 3.11 Portion of α, β and γ kafirin in 9 native sorghum flours with different pepsin treatment time

Sample	Time (hr)	Protein Fraction (%)		
		γ-Kafirin	α/β-Kafirin	α-Kafirin
HS	0.25	21.99	55.56	22.46
	0.5	21.71	56.61	21.68
	1	22.39	56.46	21.16
	2	24.98	54.98	20.05
	3	25.54	54.29	20.17
	4	26.33	53.84	19.83
HM	0.25	21.10	56.73	22.18
	0.5	21.54	56.76	21.70
	1	22.06	56.42	21.53
	2	22.65	56.40	20.95
	3	23.66	55.36	20.98
	4	23.65	55.47	20.87
HL	0.25	23.14	55.30	21.56
	0.5	20.50	57.45	22.05
	1	21.24	57.10	21.67
	2	22.79	55.85	21.36
	3	22.83	56.30	20.88
	4	23.00	56.04	20.97
MS	0.25	18.20	57.63	24.17
	0.5	18.43	57.88	23.70
	1	17.60	58.63	23.76
	2	17.91	57.67	24.43
	3	15.99	56.41	27.60

	4	22.08	55.05	22.86
MM	0.25	17.34	58.26	24.41
	0.5	17.29	58.48	24.23
	1	17.17	58.71	24.12
	2	17.99	57.64	24.38
	3	17.33	56.85	25.83
	4	21.37	55.46	23.17
ML	0.25	17.51	58.13	24.36
	0.5	16.94	58.67	24.40
	1	14.15	60.76	25.10
	2	14.84	60.25	24.91
	3	13.69	58.86	27.45
	4	17.27	58.74	23.99
SS	0.25	17.85	56.39	25.77
	0.5	22.19	52.86	24.94
	1	23.83	51.50	24.67
	2	19.67	55.72	24.60
	3	19.58	55.31	25.11
	4	20.19	55.52	24.30
SM	0.25	16.17	56.51	27.32
	0.5	21.21	54.47	24.32
	1	17.32	57.50	25.18
	2	21.53	54.48	24.00
	3	19.11	56.82	24.08
	4	20.56	56.13	23.31
SL	0.25	19.01	55.34	25.65
	0.5	20.35	55.28	24.37
	1	18.55	56.58	24.87
	2	17.00	58.11	24.88
	3	20.48	55.81	23.71



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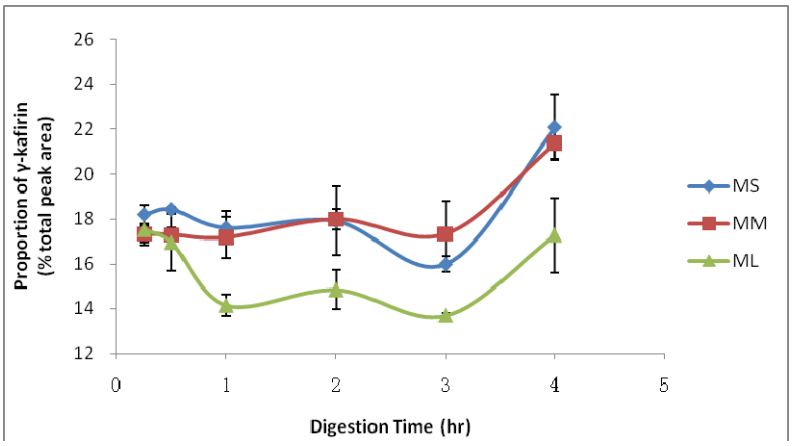

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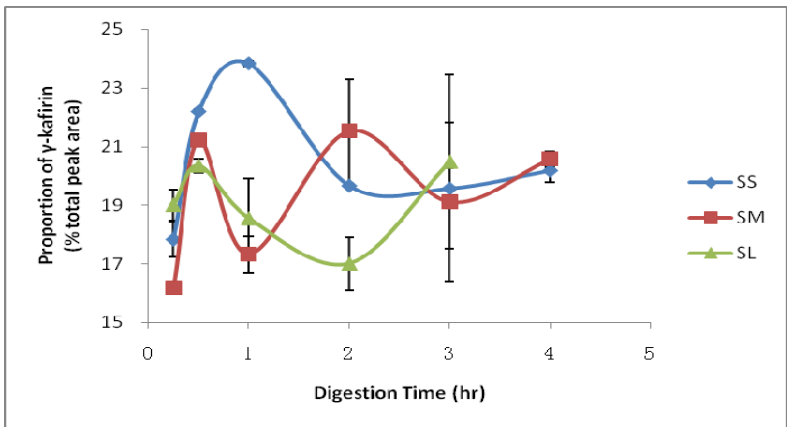
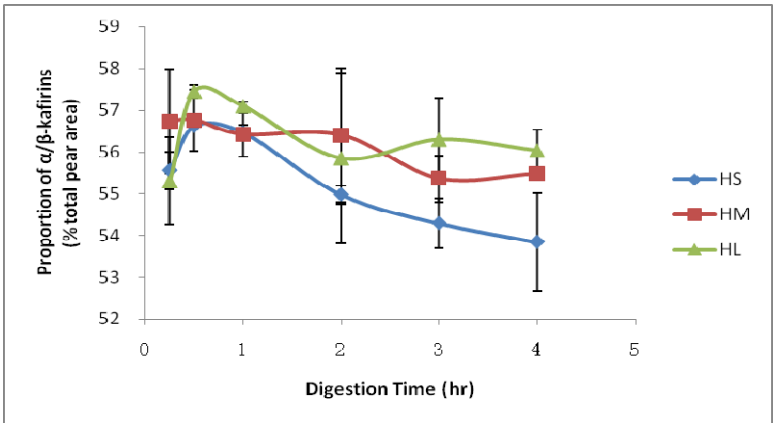


(C)

Figure 3.19 Comparison of 1st group of HPLC peak (γ-kafirin) with different pepsin treatment time in sorghum flours with different particle sizes (A: hard flour, B: median flour, C: soft flour)



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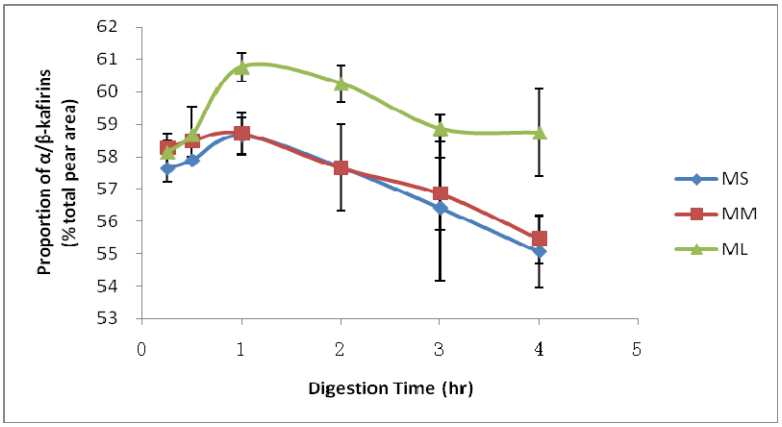

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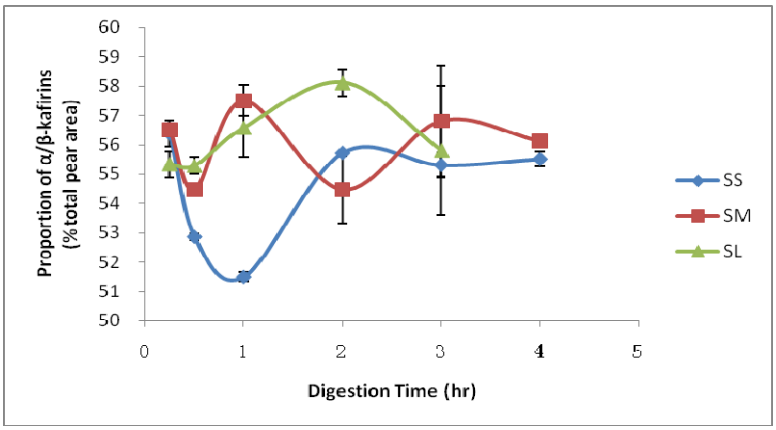
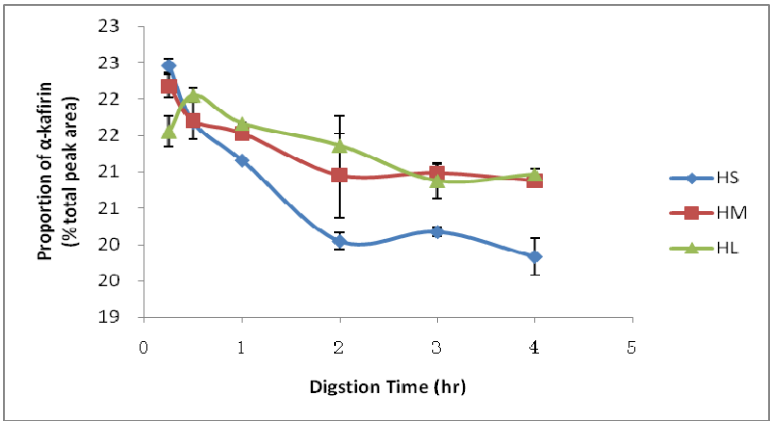


(C)

Figure 3.20 Comparison of 2nd group of HPLC peak (α/β- kafirins) with different pepsin treatment time in sorghum flours with different particle sizes (A: hard flour, B: median flour, C: soft flour).



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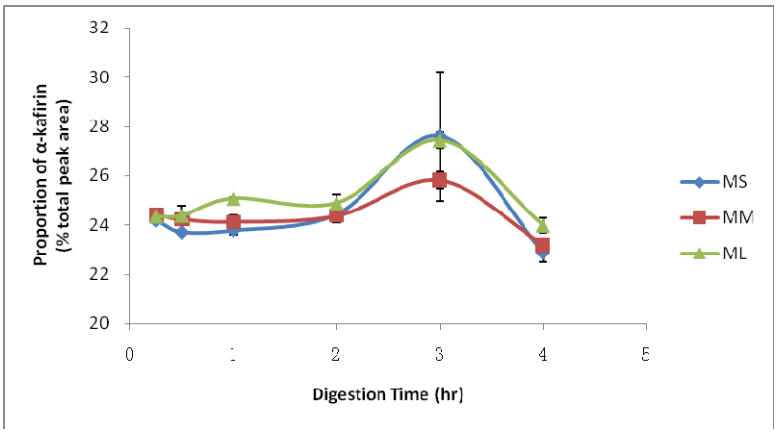

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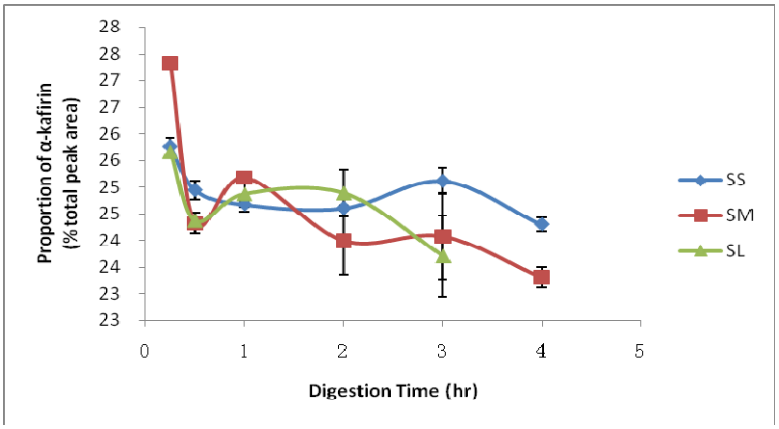
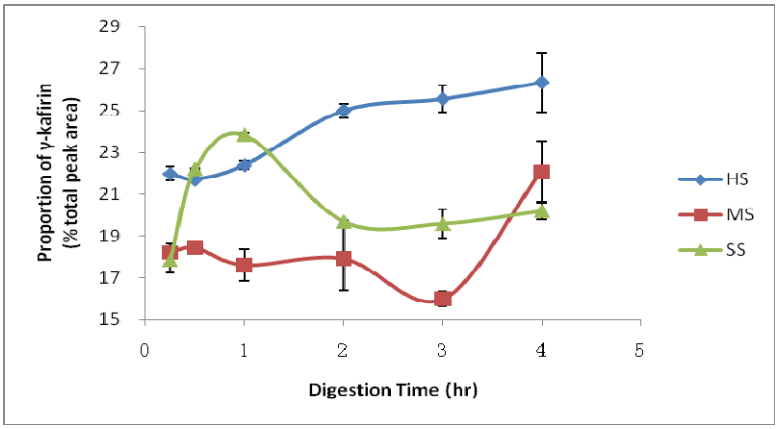


(C)

Figure 3.21 Comparison of 3rd group of HPLC peak (α-kafirin) with different pepsin treatment time in sorghum flours with different particle sizes (A: hard flour, B: median flour, C: soft flour)



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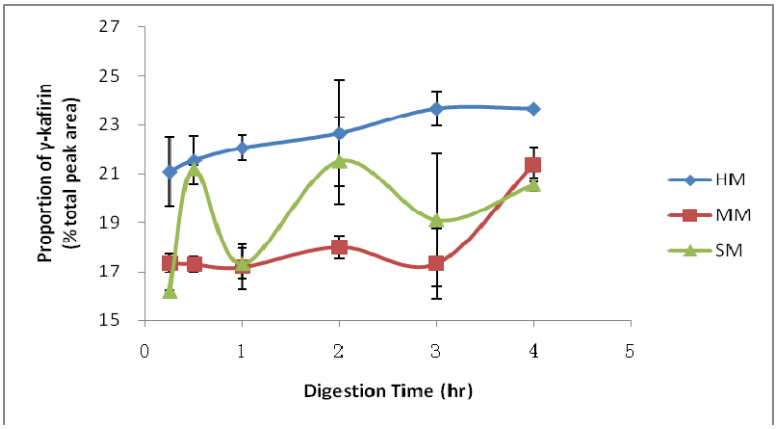

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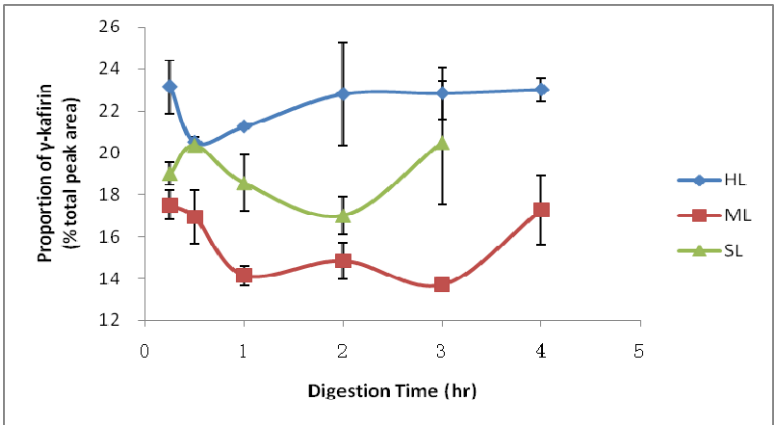
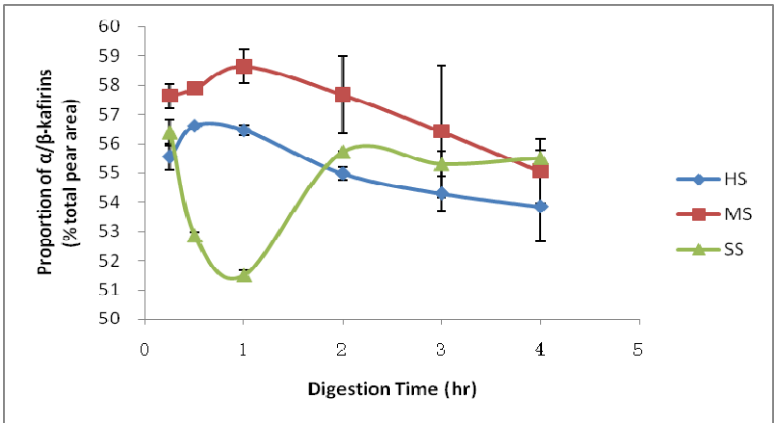


(C)

Figure 3.22 Comparison of 1st group of HPLC peak (γ-kafirin) with different pepsin treatment time in sorghum flours with different hardness (A: small particle size, B: median particle size, C: large particle size).



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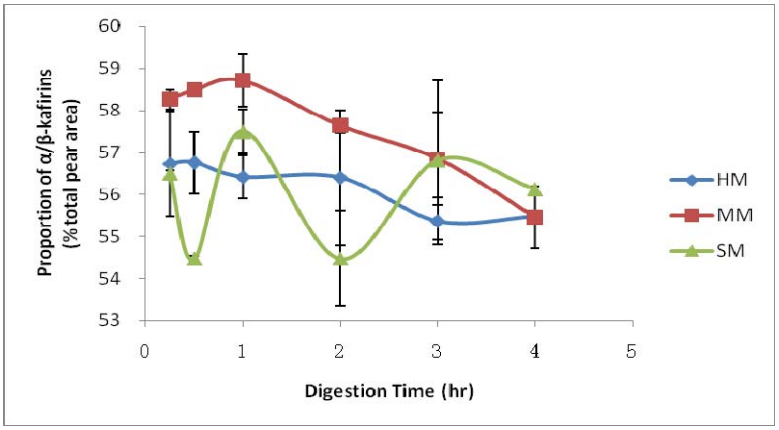

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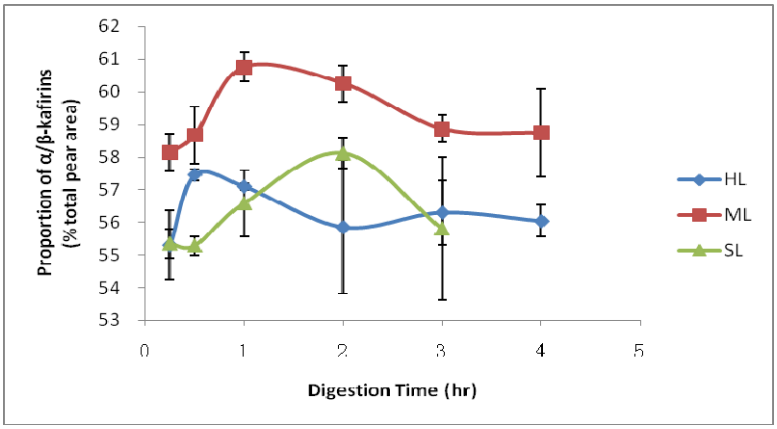
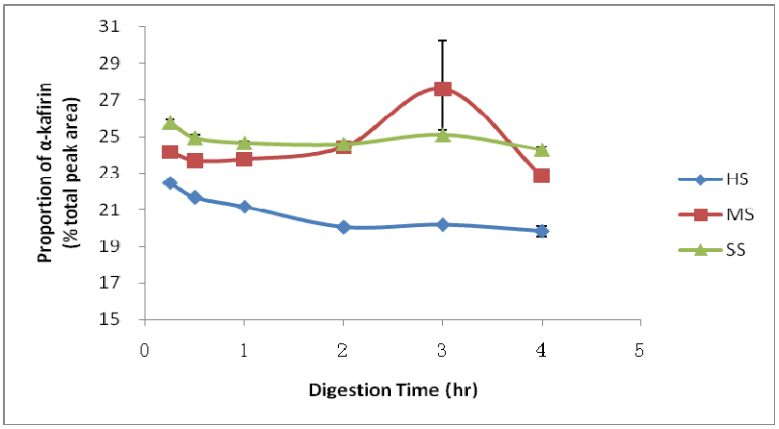


(C)

Figure 3.23 Comparison of 2nd group of HPLC peak (α/β-kafirins) with different pepsin treatment time in sorghum flours with different hardness (A: small particle size, B: median particle size, C: large particle size).



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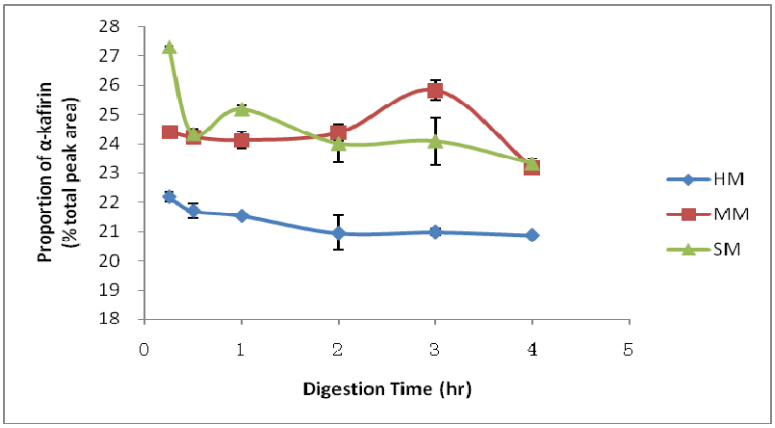

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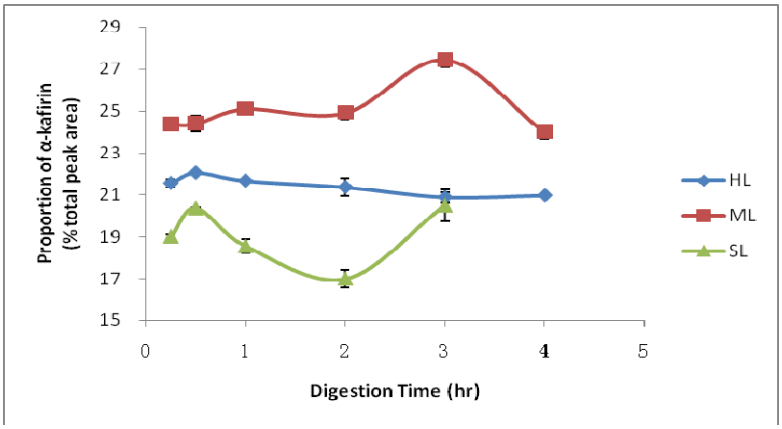
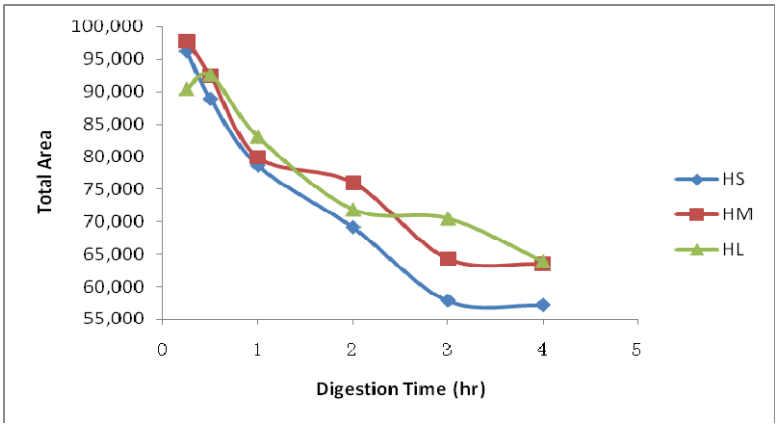


(C)

Figure 3.24 Comparison of 3rd group of HPLC peak (α-kafirin) with different pepsin treatment time in sorghum flours with different hardness (A: small particle size, B: median particle size, C: large particle size).

Table 3.12 Total area in HPLC graph of 9 native sorghum flours.

Sample	Digestion Time (hr)	Total area	Sample	Digestion Time (hr)	Total area	Sample	Digestion Time (hr)	Total area
HS	0.25	96209.632	MS	0.25	91583.013	SS	0.25	86956.978
	0.5	88899.030		0.5	85646.783		0.5	85709.989
	1	78639.035		1	74977.469		1	71506.955
	2	69131.296		2	41600.972		2	59470.140
	3	57817.445		3	30266.974		3	62893.876
	4	57138.594		4	66020.973		4	59504.144
HM	0.25	97749.152	MM	0.25	84582.612	SM	0.25	47395.099
	0.5	92393.657		0.5	85509.341		0.5	79368.455
	1	79869.487		1	71803.437		1	78775.496
	2	75973.022		2	39516.096		2	61558.438
	3	64267.903		3	37928.950		3	62894.739
	4	63450.230		4	50533.965		4	58708.389
HL	0.25	90421.611	ML	0.25	80548.342	SL	0.25	81208.330
	0.5	92582.338		0.5	81433.327		0.5	80992.934
	1	83050.606		1	77464.305		1	75066.821
	2	71874.994		2	56273.788		2	74915.375
	3	70485.890		3	49020.507		3	62158.901
	4	63906.698		4	56998.119		4	



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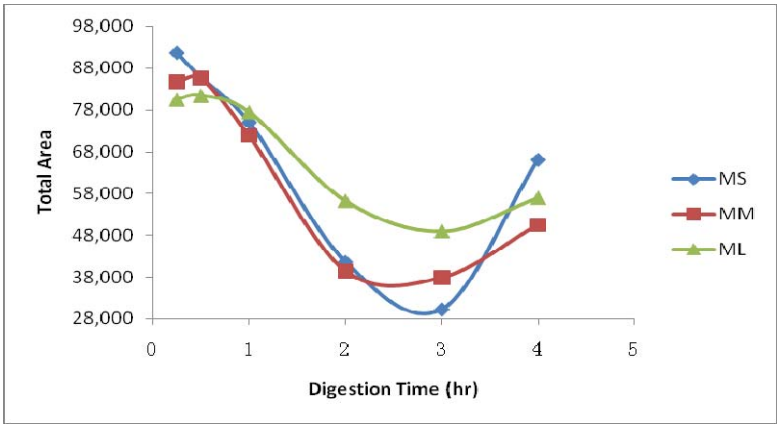

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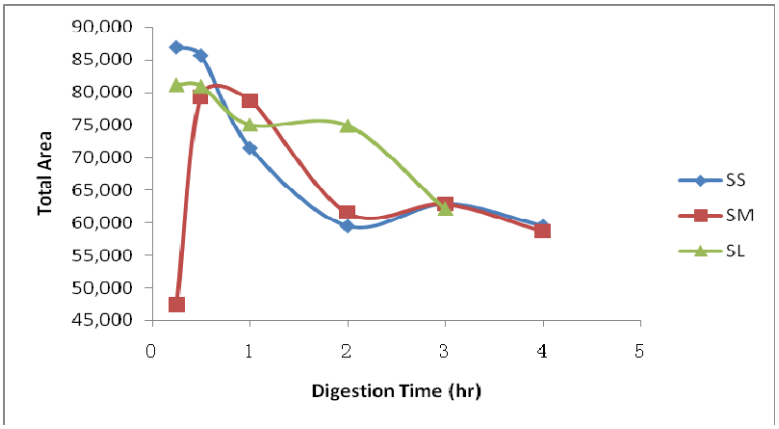
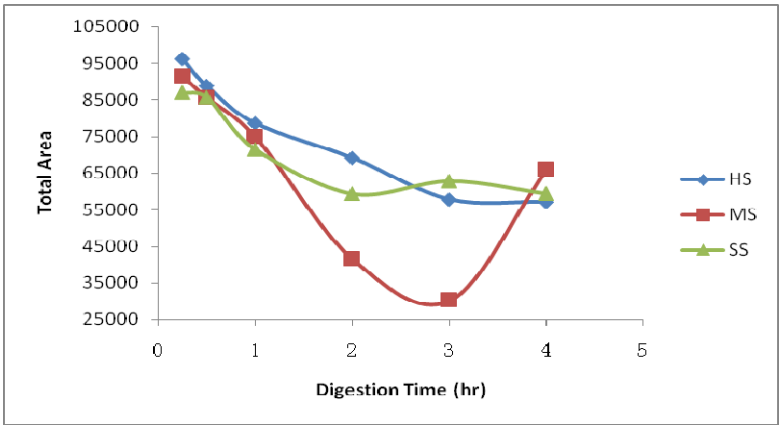
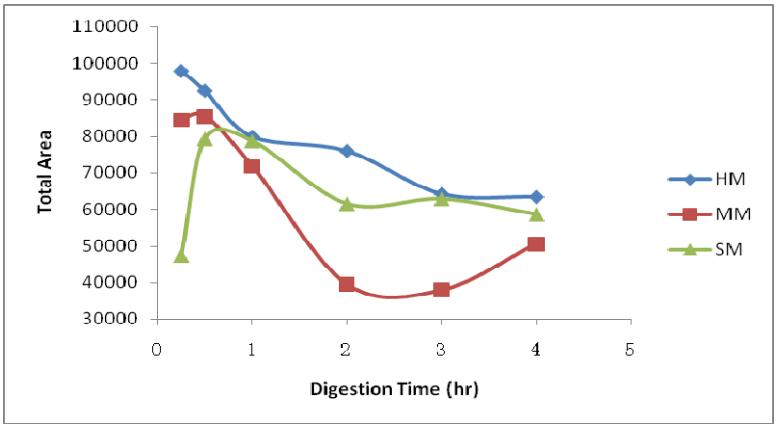


(C)

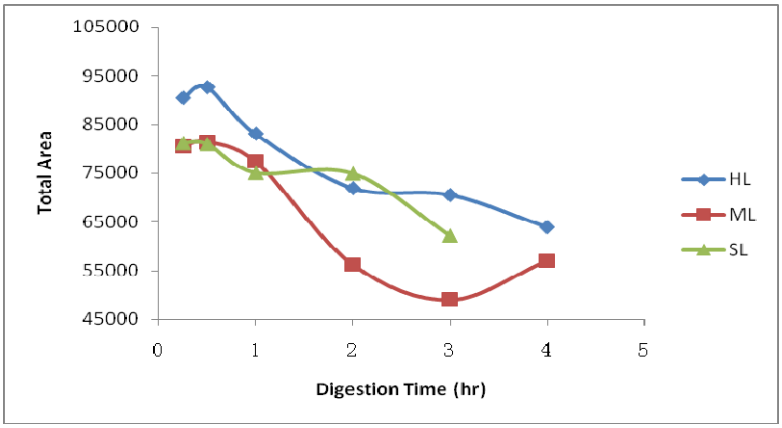
Figure 3.25 Comparison of total area in HPLC graph of same hardness sorghum flours with different particle sizes (A: hard, B: median, C: soft).



(A)



(B)



(C)

Figure 3.26 Comparison of total area in HPLC graph of same particle size sorghum flours with hardness (A: small particle size, B: median particle size, C: large particle size).

CONFOCAL MICROSCOPY

There was difference in starch digestion between samples before and after pepsin treatment (Fig. 3.27-3.33). The protein matrix was destroyed after pepsin treatment for 15 min. It was similar in the samples with pepsin treatment of 30 min, 1 hr, 2 hr, 3 hr, and 4 hr. The degree of disruption was no significant difference with different pepsin treatment time, because there was almost no protein left. The sample with 4 hr pepsin treatment swelled more than others (Fig. 3.33).

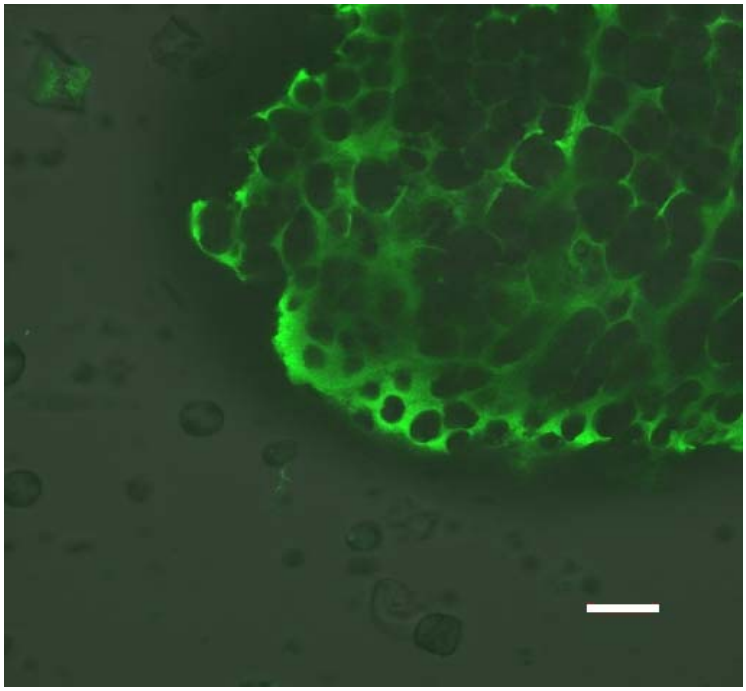


Figure 3.27 Confocal micrograph of hard sorghum flour with small particle size (HS) before pepsin treatment. Size bar = 20 µm.

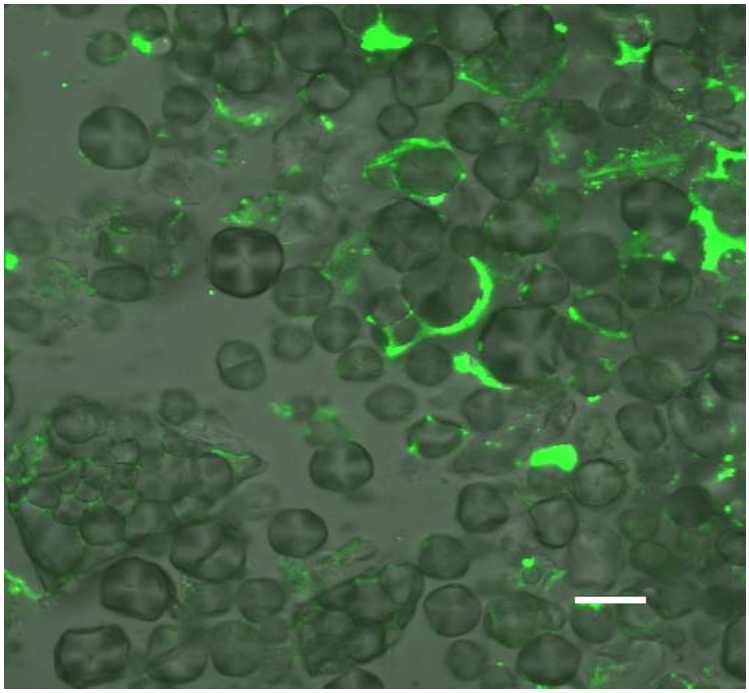


Figure 3.28 Confocal micrograph of hard sorghum flour with small particle size (HS) after 15 min pepsin digestion. Size bar = 20 µm.

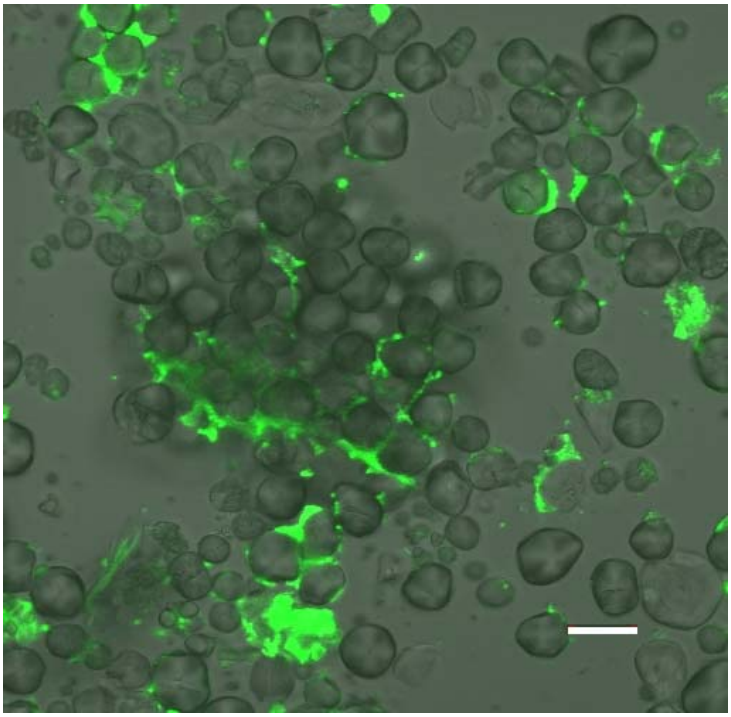


Figure 3.29 Confocal micrograph of hard sorghum flour with small particle size (HS) after 30 min pepsin digestion. Size bar = 20 µm.

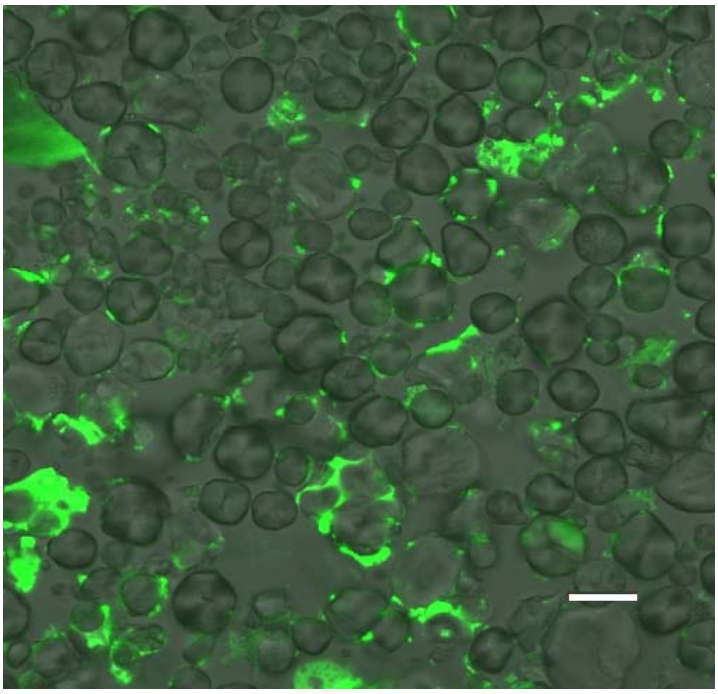


Figure 3.30 Confocal micrograph of hard sorghum flour with small particle size (HS) after 1 hr pepsin digestion. Size bar = 20 µm.

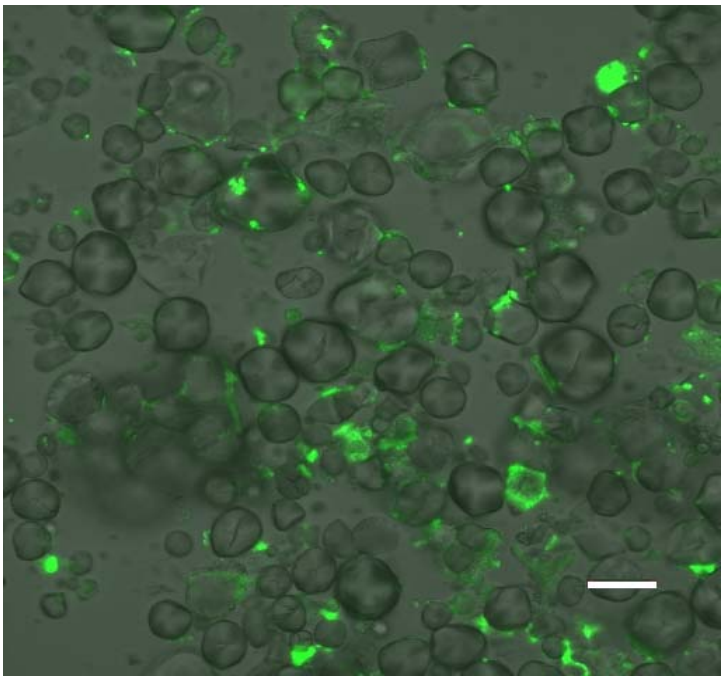


Figure 3.31 Confocal micrograph of hard sorghum flour with small particle size (HS) after 2 hr pepsin digestion. Size bar = 20 µm.

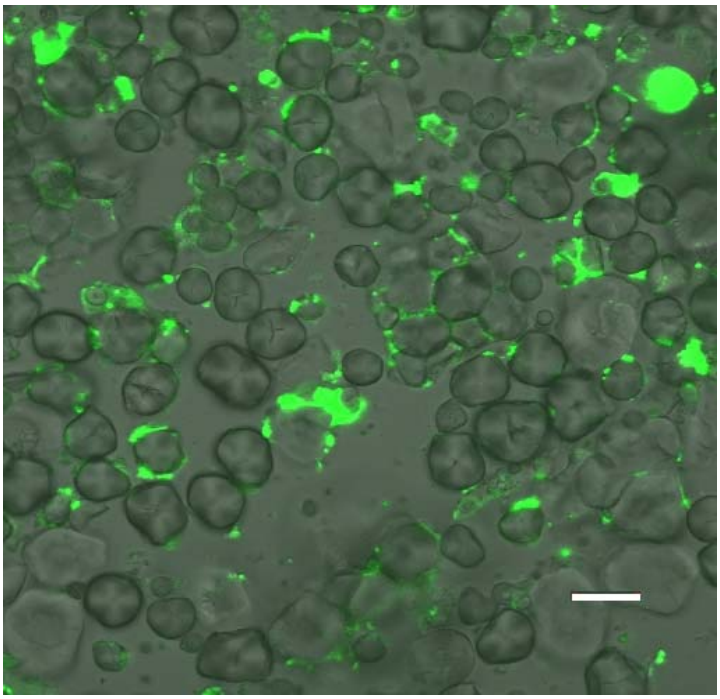


Figure 3.32 Confocal micrograph of hard sorghum flour with small particle size (HS) after 3 hr pepsin digestion. Size bar = 20 µm.

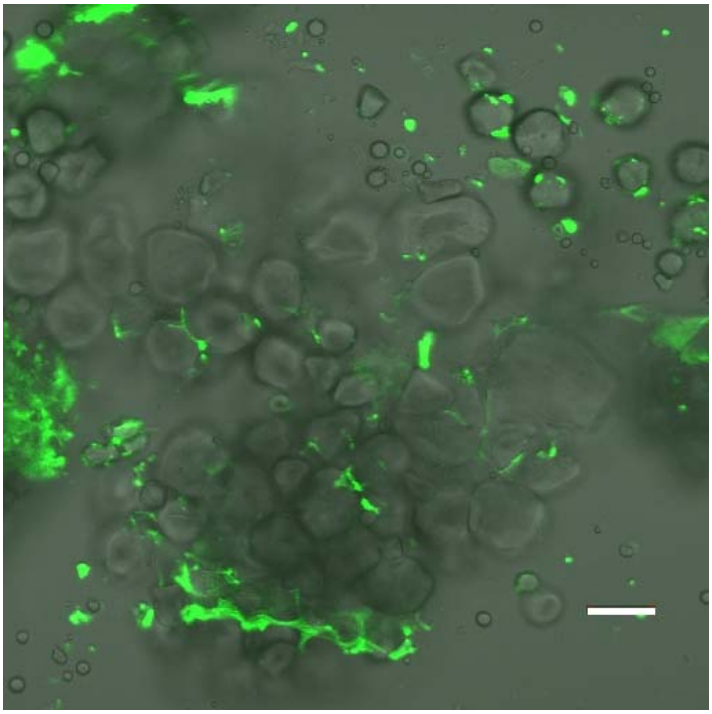


Figure 3.33 Confocal micrograph of hard sorghum flour with small particle size (HS) after 4 hr pepsin digestion. Size bar = 20 µm.

SULFHYDRYL GROUP CONTENT

Table 3.13 Free sulfhydryl group content of native and cooked sorghum samples with different hardness*

Sample	Free Sulfhydryl Group Content (nmol/mg)	
	Native	Cooked
HS	15.73±0.29	1.55±0.05
MS	14.04±0.43	1.31±0.01
SS	15.10±0.09	0.97±0.10

* Values are means±SD and are expressed as nmol/mg of protein.

Sulfhydryl group content decreased after cooking (Table 3.13), indicating that there were disulfide bonds formed during heating. The disulfide bonds connected the proteins in sorghum flour. The cross-linked protein strengthened the structure of protein matrix in sorghum flour. Digestibility of starch in cooked sorghum flour was lower compared to that of starch in cooked corn flour (Lewis et al, 2008) and wheat flours. However, in my work, digestibility of starch in cooked sorghum flours was higher than that of starch in un-cooked sorghum flours. Cooking gelatinized starch in sorghum, making it more susceptible to enzyme digestion.

CHAPTER 4 - CONCLUSIONS

1. Digestion of starch in sorghum was affected by the protein matrix. Low protein digestibility at pH 1.3 led to a low digestion of starch. When pH 2.0, optimum pH for pepsin, was used, higher protein digestibility was achieved, and led to higher starch digestibility. Protein hydrolysis increased with the time of pepsin treatment, leading to increased starch digestion. Confocal microscopy showed the protein matrix was disrupted after pepsin treatments, leading to the increased digestion of starch.
2. RDS content of isolated sorghum starch was about 20-40%, SDS content was 51-60%, and RS was approximately 9-20%. They are similar with that of normal maize starch, which has a relative low digestibility among cereal starches.
3. Amylose content was higher in sorghum flour with median hardness than that in hard and soft flours, causing lower starch digestibility.
4. Protein digestibility decreased after cooking while starch digestibility increased compared to native sorghum flours. Sulfhydryl groups decreased after cooking, indicating the disulfide bonds formed between protein molecules and increased barrier for enzyme to digest starch.

Future work is needed: determine particle size of sorghum flour after pepsin treatment; determine proximate composition of the 9 sorghum flours including ash, fat, and fiber; determine the RS level in starches with less than 0.3% protein; use confocal laser scanning microscopy to examine cooked sorghum flours after pepsin treatment.

References

AACC International. 2000. Approved Methods of the American Association of Cereal Chemists, 10th Ed. Methods 44-15A and 46-30. The Association: St. Paul, MN.

Aarathi, A., Urooj, A., and Puttaraj, S. 2003. In vitro starch digestibility and nutritionally important starch fractions in cereals and their mixtures. Starch. 55:94-99.

Aboubacar, A., Axtell, J. D., Huang, C. P., and Hamaker, B. R. 2001. A rapid protein digestibility assay for identifying highly digestible sorghum lines. Cereal Chem. 78:160-165.

Asp, N.G. 1992. Resistant starch – proceedings from the second plenary meeting of EURESTA: European FLAIR Concerted Action No. 11 on physiological implications of the consumption of resistant starch in man. Preface Eur. J. of Clin. Nutr. 46:S1.

Axtell, J. D., Kirleis, A., Hassan, M. M., D'Croz, Mason, N., Mertz, E. T., and Munck, L. 1981. Digestibility of sorghum proteins. Proc. of the Nat. Acad. of Sci. U. S. A. 78:1333-1335.

Bantan-Polak, T., Kassai, M., and Grant, K. B. 2001. A comparison of fluorescamine and naphthalene-2,3-dicarboxaldehyde fluorogenic reagents for microplate-based detection of amino acids. Anal. Biochem. 297:128-136.

Bean, S. R., Chung, O. K., Tuinstra, M. R., Pedersen, J. F., and Erpelding, J. 2006. Evaluation of the single kernel characterization system (SKCS) for measurement of sorghum grain attributes. Cereal Chem. 83:108-113.

Bean, S. R., Lookhart, G. L., and Bietz, J. A. 2000. Acetonitrile as a Buffer Additive for Free Zone Capillary Electrophoresis Separation and Characterization of Maize (Zea mays L.) and Sorghum (Sorghum bicolor L. Moench) Storage Proteins. J. Agric. Food Chem. 48:318-327.

Benmoussa, M., Suhendra, B., Aboubacar, A., and Hamaker, B. R. 2006. Distinctive sorghum starch granule morphologies appear to improve raw starch digestibility. Starch. 58:92-99.

Buleon, A., Colonna, P., Planchot, V. and Ball, S. 1998. Starch granules: structure and biosynthesis. Int. J. Biol. Macromol. 23:85-112.

Burns, A. J., and Rowlands, I. R. 2000. Anti-carcinogenicity of probiotics and prebiotics. Current Issues in Intestinal Microbiology. 1:13-24.

Chan, K.-Y., and Wasserman, B. P. 1993. Direct colorimetric assay of free sulfhydryl and disulfide bonds in suspensions of solubilized and particulate cereal proteins. Cereal Chem. 70:22-26.

Chandrashekar, A., and Kirleis, A. W. 1988. Influence of protein on starch gelatinization in sorghum. Cereal Chem. 65:457-462.

Chibber, B. A. K., Mertz, E. T., and Axtell, J. D. 1980. In vitro digestibility of high tannin sorghum at different stages of dehulling. J. Agric. Food Chem. 28:160-161.

Choi, S. J., Woo, H. D., Ko, S. H., and Moon, T. W. 2008. Confocal laser scanning microscopy to inverstigate the effect of cooking and sodium bisulfate on in vitro digestibility of waxy sorghum flour. Cereal Chem. 85:65-69.

Cornish-Bowden, A. and Wharton, C. W. 1988. Simple enzyme kinetics. In Rickwood, D. (ed.), Enzyme Kinetics. IRL Press, Washington, DC. pp:1-18.

Davis, E. A. 1994. Wheat starch. Cereals Food World. 39:34-36.

Dietary Guidelines for Americans. 2005. http://www.health.gov/dietaryguidelines/dga2005/document/html/chapter7.htm.

Duodu, K. G., Taylor, J. R. N., Belton, P. S., and Hamaker, B. R. 2003. Factors affecting sorghum protein digestibility. J. Cereal Sci. 38:117-131.

Duodu, K. G., Nunes, A., Delgadillo, I., Parker, M. L., Mills, E. N. C., Belton, P. S., and Taylor, J. R. N. 2002. Effect of grain structure and cooking on sorghum and maize in vitro protein digestibility. J. Cereal Sci. 35:161-174.

Elkin, R. G., Arthur, E., Hamaker, B. R., Axtell, J. D., Douglas, M. W., and Parsons, C. M. 2002. Nutritional value of a highly digestible sorghum cultivar for meat-type chickens. J. Agric. Food Chem. 50:4146-4150.

Elmalik, M. 1985. Studies on the nutritional quality of sorghum grain. Ph. D. dissertation, Kansas State University, Manhattan, KS.

Englyst, K. N., Englyst, H. N., Hudson, G. J., Cole, T. J., and Cummings, J. H. 1999. Rapidly available glucose in foods. An in vitro measurement that reflects the glycemic response. Am. J. Clin. Nutr. 69:448-454.

Englyst, K. N., Vinoy, S., Englyst, H. N., and Lang, V. 2003. Glycaemic index of cereal products explained by their content of rapidly and slowly available glucose. Br. J. Nutr. 89:329-339.

Englyst, H. N., Kingman, S. M., and Cummings, J. H. 1992. Classification and measurement of nutritionally important starch fractions. Euro. J. Clinc. Nutri. 46:S33-S50.

Englyst, H. N., Wiggins, H. S., and Cummings, J. H. 1982. Determination of the non-starch polysaccharides in plant foods by GasLiquid chromatography of constituent sugars as alditol acetates. Analyst. 107:307-318.

Ezeogu, L. I., Duodu, K. G., Emmambux, N. M., and Taylor, J. R. N. 2008. Influence of cooking conditions on the protein matrix of sorghum and maize endosperm flours. Cereal Chem. 85:397-402.

Ezeogu, L. I., Duodu, K. G., and Taylor, J. R. N. 2005. Effects of endosperm texture and cooking conditions on the in vitro starch digestibility of sorghum and maize flours. J. Cereal Sci. 42:33-44.

Ferguson, L. R., Tasman-Jones, C., Englyst, H. N., and Harris, P. J. 2000. Comparative effects of three resistant starch preparations on transit time and short-chain fatty acid production in rats. Nutr. Cancer. 36:230-237.

Goddard, M. S., Young, G., and Marcus, R. 1984. The effect of amylase content on insulin and glucose responses to ingested rice. Am. J. Clin. Nutr. 39:388-392.

Hale, W. H. 1980. Feed preparation and processing. In: D. C. Church (Ed.) Digestion Physiology and Nutrition of Ruminants, Vol. 3 (2nd Ed.). pp 19--35. O & B Books, Inc., Corvallis, OR.

Hamaker, B. R., and Bugusu, B. A. 2003. Overview: Sorghum proteins and food quality. Paper 8 in: Afripro Workshop on the Proteins of Sorghum and Millets. P. S. Belton and J. R. N. Taylor, eds. Available online at www.afripro.org.uk.

Hamaker, B. R., Kirleis, A. W., Mertz, E. T., and Axtell, J. D. 1986. Effect of cooking on the protein profiles and in vitro digestibility of sorghum and maize. J. Agric. Food Chem. 34:647-649.

Hamaker, B. R., Mohamed, A. A., Habben, J. E., Huang, C. P., and Larkins, B. A. 1995. Efficient procedure for extracting maize and sorghum kernel proteins reveals higher prolamin contents than the conventional method. Cereal Chem. 72:583-588.

Han, X. Z., and Hamaker, B. R. 2002. Location of starch granule-associated proteins revealed by confocal laser scanning microscopy Journal of Cereal Science 35:109-116.

Han, X. Z., Benmoussa, M., Gray, J. A., BeMiller, J. N., and Hamaker, B. R. 2005. Detection of proteins in starch granule channels. Cereal Chem. 82:351-355.

James, M. G., Denyer, K., and Myers, A. M. 2003. Starch synthesis in the cereal endosperm. Curr. Opin. Plant Biol. 6:215-222.

Jenkins, D. J., Wolever, T. M., and Taylor, R. H. 1981. Glycemic index of foods: a physiological basis for carbohydrate exchange. Am. J. Clin. Nutr. 34:362-366.

Lawrence, T.L.J. 1970. Some effects of including differently processed barley in the diet of the growing pig. 1. Growth rate, food conversion efficiency, digestibility and rate of passage through the gut. Anita. Prod. 12:139.

Leach, H. W. 1965. Gelatinization of starch. in Starch, Chemistry and Technology, Academic Press, New York.

Li, M., Kim, J. W., and Peeples, T. L. 2002. Kinetic enhancement of starch bioconversion in thermoseparating aqueous two-phase reactor systems. J. Biochem. Engng. 11:25-32.

Lichtenwalner, R. E., Ellis, E. B., and Rooney, L. W. 1978. Effect of incremental dosages of the waxy gene of sorghum on digestibility. J. Anim. Sci. 46:1113-1119.

Luce, W. G., L. T. Omtvedt and D. G. Stephens. 1970. Grinding and dry-roUing of milo and wheat for growing-finishing swine. Oklahoma Agr. Exp. Sta. Misc. Pub. MP-84:60.

Ludwig, D. S. 2000. Dietary glycemic index and obesity. J. Nutr. 130:280S-283S.

Nichols, B. L., Avery, S., Sen, P., Swallow, D. M., Hahn, D., and Sterchi, E. 2003. The maltase-glucoamylase gene: common ancestry to sucrase-isomaltase with complementary starch digestion activities. Proc. Natl. Acad. Sci. U. S. A. 100:1432-1437.

Oates, C. G. 1997. Towards an understanding of starch granule structure and hydrolysis. Trends Food Sci. Technol. 8:375-382.

Oria, M. P., Hamaker, B. R., and Shull, J. M. 1995. Resistance of sorghum α-, β- and γ-kafirins to pepsin digestion. J. Agric. Food Chem. 43:2148-2152.

Oria, M. P., Hamaker, B. R., Axtell, J. D., and Huang, C. P. 2000. A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm protein bodies. Proc. Natl. Acad. Sci. 97:5065-5070.

Owsley, W. F., Knabe, D. A., and Tanksley, T. D. 1981. Effect of sorghum particle size on digestibility of nutrients at the terminal ileum and over the total digestive tract of growing-finishing pigs. J. Anim. Sci. 52: 557-566.

Planchot, V., Colonna, P., and Buleon, A. 1997. Enzymatic hydrolysis of α-glucan crystallites. Carbohydr. Res. 298:319-326.

Rooney, L. W. and Miller, F. R. 1982. Variation in the structure and kernel characteristics of sorghum. Proc. Sorghum Grain Quality Syrup. pp 143-161. Int. Crops Res. Inst. for the Semi-arid Tropics, Hyderabad, India

Rooney, L. W. and Pflugfelder, R. L. 1986. Factor affecting starch digestibility with special emphasis on sorghum and corn. J. Anim. Sci. 63:1607-1623.

Rusnak, B. A., Chou, C. L., and Rooney L. W. 1980. Effect of micronizing on sorghum varieties with different endosperm type. J. Food Sci. 45: 1529-1534.

Schlamowitz, M. and Peterson, L. U. 1959. Studies on the optimum pH for the action of pepsin on "native" and denatured bovine serum albumin and bovine hemoglobin. J. Biol. Chem. 234:3137-3145.

Shin, S. I., Choi, H. J., Chung, K. M., Hamaker, B. R., Park, K. H., and Moon, T. W. 2004. Slowly digestible starch from debranched waxy sorghum starch preparation and properties. Cereal Chem. 81:404-408.

Shull, J. M., Watterson, J. J., and Kirleis, A. W. 1991. Proposed nomenclature for the alcohol-soluble proteins (kafirins) of Sorghum bicolor (L. Moench) based on molecular weight, solubility, and structure. J. Agric. Food Sci. 39:83-87.

Shull, J. M., Watterson, J. J., and Kirleis, A. W. 1992. Purification and immunocytological localization of kafirins in Sorghum bicolour endosperm. Protoplasma. 171:64-74.

Sierks, M. R., Ford, C., Reilly, P. J., and Svensson, B. 1990. Catalytic mechanism of fungal glucoamylase as defined by mutagenesis of Asp176, Glu179 and Glu180 in the enzyme from *Aspergillus awamori*. Protein Engng. 3:193-198.

Sikabbubba, R. 1989. The effect of alcohol soluble proteins on the digestibility of sorghum. Ph. D. dissertation, Kansas State University, Manhattan, KS.

Slaughter, S. L., Ellis, P. R., and Butterworth P. J. 2001. An investigation of the action of porcine pancreatic alpha-amylase on native and gelatinised starches. Biochimic et Biophysica Acta. 1525:29-36.

Sullins, R. D. and Rooney, L. W. 1975. Light and scanning electron microscopic studies of waxy and non-waxy endosperm sorghum varieties. Cereal Chem. 52:361-366.

Sullins, R. D., Rooney, L. W., and Riggs, J. K. 1971. Physical changes in the kernel during reconstitution of sorghum grain. Cereal Chem. 48:567-575.

Tester, R. F., Karkalas, J., and Qi, X. J. 2004. Starch - composition, fine structure and architecture. J. Cereal Sci. 39:151-165.

Tetlow, I. J., Morell, M. K., and Emes, M. J. 2004. Recent developments in understanding the regulation of starch metabolism in higher plants. J. Exp. Bot. 55:2131-2145.

Topping, D. L. and Clifton, P. M. 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and non-starch polysaccharides. Physiology Reviews. 81:1031-1064.

Theurer, C. B. 1986. Grain processing effects on starch utilization by ruminants. J. Anim. Sci. 63:1649-1662.

Vanhoof, K. and De Schrijver, R. 1998. The influence of enzymeresistant starch on cholesterol metabolism in rats fed on a conventional diet. Bri. J. Nutr. 80:193-198.

Voragen, A. G. J. 1998. Technological aspects of functional food related carbohydrates. Trends in Food Sci. Technol. 9:328-335.

Watson, S. A., Sanders, E. H., Wakely, R. D., and Williams, C. B. 1955. Peripheral cells of the endosperms of grain sorghum and corn and their influence on starch. Cereal Chem. 32:165-182.

Watterson, J. J., Shull, J. M., and Kirleis, A. W. 1993. Quantitation of α-, β-, and γ-kafirins in vitreous and opaque endosperm of sorghum bicolor. Cereal Chem. 70: 452-457.

Williams, M. R. and Bowler, P. 1982. Starch gelatinization – a morphological study of Triticeae and other starches. Starch. 34:221-223.

Wilson, J. D., Bechtel, D. B., Todd, T. C., and Seib, P. A. 2006. Measurement of wheat starch granule size distribution using image analysis and laser diffraction technology. Cereal Chem. 83: 259-268.

Wollowski, I., Rechkemmer, G., and Pool-Zobel, B. L. 2001. Protective role of probiotics and prebiotics in colon cancer. Am. J. of Clin. Nutr. 73:451S-455S.

Zhang, G., and Hamaker, B. R. 1998. Low α-amylase starch digestibility of cooked sorghum flours and the effect of protein. Cereal Chem. 75:710-713.

Zhang, G., Ao, Z., and Hamaker, B. R. 2006a. Slow digestion property of native cereal starches. Biomacromolecules.7:3252-3258.

Zhang, G., Venkatachalam, M., and Hamaker, B. R. 2006b. Structural basis of the slow digestion property of native cereal starches. Biomacromolecules. 7:3259-3266.