PROCESSING AND CHARACTERIZATION OF SORGHUM PROTEIN CONCENTRATES
USING EXTRUSION-ENZYME LIQUEFACTION

by

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B.S., University of the Philippines, 2000
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
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# Abstract

Sorghum grain (*Sorghum bicolor*) is safe for consumption by individuals afflicted with celiac disease, and its proteins can be used as a supplement in gluten-free foods. However, utilization of sorghum in human foods is limited by the poor digestibility and lack of functionality of its proteins, which result from their entrapment in protein bodies, tight association with starch, and high degree of cross-linking induced by cooking. The first part of this study presents an extensive review of current methods for concentration and isolation of sorghum proteins, which are laboratory-scale techniques used for protein characterization and have no potential for commercial scale-up. Furthermore, these methods typically use non-food grade reagents and do not improve protein digestibility and functionality. In the second part, a novel extrusion-enzyme liquefaction (EEL) process was used to produce sorghum protein concentrates to overcome the aforementioned limitations. EEL involves extrusion pre-treatment of sorghum flour and starch liquefaction with a thermostable α-amylase, followed by enzyme inactivation, protein separation and drying. To demonstrate the concept, a laboratory-scale EEL process was used to produce concentrates with higher protein content (PC; 80% db) and digestibility (D; 74%) than those made by batch liquefaction. The optimum conditions for producing concentrates with both high PC and D were 32% wb in-barrel moisture content and 2.5% α-amylase added after extrusion. Using these conditions, EEL was scaled-up to a pilot-scale process to produce sorghum protein concentrates with 72-80% db PC and 62-74% D, while the batch liquefied control had only 70% db PC and 57% D. Dynamic oscillatory measurements of dough (55% moisture) and batter (65% moisture) containing sorghum protein concentrates (5 and 10%) and potato starch were performed to evaluate protein functionality. At lower moisture, pure potato starch and dough containing 10% sorghum protein concentrate had similar elastic and viscous moduli. At higher moisture, potato starch was more stable and exhibited significantly higher moduli than the batters with protein concentrates. Sorghum protein concentrates can improve the quality of some gluten-free foods. EEL shows promise for commercial production of sorghum protein concentrates because of its high throughput and potential for delivering high protein content and digestibility.

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# Dedication

The one Almight God, Jesus Christ and the Holy Spirit

The Blessed Virgin Mary

# Chapter 1- Introduction

In the United States, sorghum is used largely in animal feed but its use in human food is scant.  Given that almost 40% of its worldwide production is for human consumption (Dendy 1995; Rooney and Waniska 2000; Engleson and Atwell 2008), sorghum has a huge potential for use in human foods in the U.S., especially in gluten-free products.  A market study quoted by Engleson and Atwell (2008) reported an 86% growth in gluten-free product introductions in 2006.  Mintel (2007) reported that the gluten-free foods and beverages market in 2006 was worth $700 Million, and it was projected to grow annually at a rate of 15-25% to $1.3 billion in 2010. Bakery goods segment had the most number of new gluten-free introductions in 2006 (22% of total).  The large market growth is due to increasing awareness of celiac disease, a condition whereby an individual suffers a variety of gastrointestinal and non-gastrointestinal symptoms as a result of gluten ingestion.  This disease affects 1 in 133 or roughly 3 million Americans, and the only treatment is a lifelong gluten-free diet (Fasano and others 2003).  The demand for gluten-free foods will be even higher because around 97% of those afflicted with the disease are still undiagnosed.

Celiac patients are highly vulnerable and typically suffer from malnutrition due to poor nutrient absorption in the small intestine that has been seriously ravaged through years of gluten ingestion.  Additionally, because celiac disease is often diagnosed during adulthood, the transition of patients to a gluten-free diet is a difficult experience.  The food choices of a celiac patient's family and friends are also seriously affected.  High quality and nutritious foods that are acceptable to both celiacs and the people around them are demanded, and this concern has to be treated with utmost immediacy.

While sorghum is safe for celiac patients (Ciacci and others 2007) and is neutral in flavor and color, its use in foods faces several challenges.  First, sorghum proteins have low digestibility, which is further reduced during cooking with high moisture (Oria and others 1995; Duodu and others 2002; Ezeogu and others 2008).  Second, these proteins have limited functionality and do not possess the rheological characteristics of wheat gluten, making it difficult to mimic the eating qualities of wheat-based products (Engleson and Atwell 2008). These limitations are primarily due to the morphology of sorghum proteins, that is, they are tightly bound in spherical protein bodies that are very resistant to disruption (Duodu and others

2003). The huge gap existing between the present and desired nutritional and functional characteristics of sorghum proteins needs to be addressed in order to increase sorghum consumption in foods.


## 1.1 THEORETICAL FRAMEWORK

A sorghum protein concentrate with improved protein content and digestibility can be produced by extrusion cooking and starch liquefaction by a thermostable α-amylase. The high temperature and shear conditions during extrusion cooking are known to gelatinize and degrade starch (Lai and Kokini 1991; Chinnaswamy and Hanna 1990; Diosady and others 1985), making it readily available for α-amylase degradation. Additionally, extrusion cooking has been documented to improve *in vitro* protein digestibility of sorghum proteins (Hamaker and others 1994; Dahlin and Lorenz 1993; Fapojuwo and others 1987). This method of processing has also been documented to disrupt the protein bodies of corn, which are highly homologous to sorghum protein bodies (Batterman-Azcona and others 1999 a, b). A similar mechanism of protein disruption is expected to result in the extrusion of sorghum proteins. The theoretical framework of this study is summarized in the figure below (Fig. 1.1).


## 1.2 GENERAL OBJECTIVE

The overarching goal of this study was to increase sorghum utilization through the production of a protein concentrate which was thermo-mechanically modified for nutritional and functional improvement of foods. It was envisioned that the process developed would have a potential for commercial scale-up. The potential to grow the sorghum market for human foods relies primarily on the acceptability and nutritional value of sorghum products, which are dependent on the properties of sorghum proteins.

## 1.3  SPECIFIC OBJECTIVES

1. To review existing processes for concentrating and isolating sorghum protein, particularly kafirins (Chapter 2);

2. To demonstrate the feasibility of producing sorghum protein concentrates by extrusion-enzyme liquefaction and to recommend optimum processing conditions based on laboratory-scale studies (Chapter 3);

3. To scale-up the extrusion-enzyme liquefaction process and systematically study key processing conditions for producing sorghum protein concentrates with high purity and digestibility (Chapter 4);

4. To characterize the dynamic rheological properties of sorghum protein concentrates in a model system and to relate these properties to real dough and batter food systems (Chapter 5)
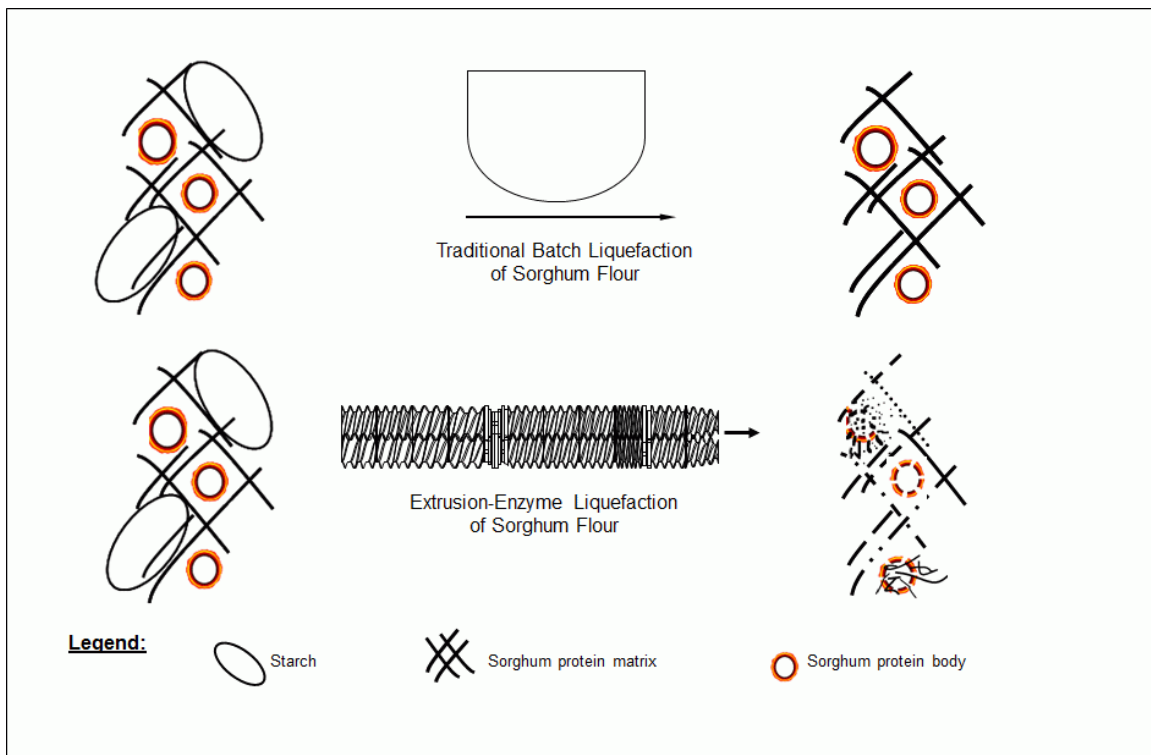


**Fig. 1.1  Hypothesized Mechanism for the Extrusion-Enzyme Liquefaction Process in Comparison to Traditional Batch Liquefaction.**

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# Chapter 2 - Sorghum Proteins:  The concentration, isolation, modification, and food applications of kafirins[1]

## 2.1  ABSTRACT

Celiac disease is a serious condition affecting millions of individuals.  Those afflicted with this illness are resigned to a lifelong avoidance of products containing the storage prolamin proteins found in cereal grains wheat, rye and barley.  Since many food products are based on these cereals, especially wheat, celiac patients have very limited food choices; and, those that are available to them are generally poor in quality, often nutritionally deficient and expensive.  Furthermore, this condition also indirectly affects their families and friends with whom they share meals.  Thus, a burgeoning need exists to develop nutritious, palatable and affordable foods, especially staples like bread and pasta, for these individuals and their families and friends who are accustomed to wheat based products.  Grain sorghum and its proteins are safe for celiac patients and individuals with varying levels of gluten intolerances.  However, the main sorghum proteins, kafirins, are resistant to digestion. They are also difficult to extract and modify in an industrial-scale process and with food-compatible chemicals, thus limiting their use in foods. This review describes studies on kafirin extraction and methods for modifying sorghum proteins for improved nutrition and functionality, as well as food applications.  Armed with this knowledge, scientists and technologists will be in a better position to identify opportunities that will further enhance the nutritional and functional value of sorghum proteins.

## 2.2  INTRODUCTION

### 2.2.1  *Gluten intolerance is a serious and prevalent issue*

Prolamin proteins in the cereal grains wheat (gluten), rye (secalin) and barley (hordein), are known to bring about an allergic response or a detrimental autoimmune reaction in certain individuals.  The latter, a condition called celiac disease (CD), afflicts 1 in 133 Americans (Fasano and others 2003).  CD (also referred to as celiac sprue, nontropical sprue and gluten-sensitive enteropathy) is a chronic, genetic disease characterized by the formation of autoantibodies and the destruction of the mucosal lining of the small intestine, which results in nutrient malabsorption.  Typical symptoms associated with CD are abdominal pain, diarrhea, and constipation.  Long term complications of this disease include anemia, osteoporosis, miscarriage, liver diseases, cancers of the intestine and depression or anxiety (NIDDK 2009).  In many individuals, the disease becomes evident only during adulthood, and is sometimes triggered after

surgery, pregnancy, childbirth, viral infection or severe emotional stress. For this reason, even though 1% of the U.S. population is thought to be afflicted with CD, about 97% of these cases are undiagnosed (Mintel 2007). A life-long avoidance of products containing gluten, secalin and hordein is the only treatment. Additionally, because gluten is found not only in foods but also in medicines, vitamins, beauty products, stamps and envelope adhesives, celiacs have to be exceedingly judicious.

### 2.2.2   Market research shows a heightened demand for gluten-free products

Increased awareness and diagnosis of CD and gluten sensitivity have spurred the demand for gluten-free products. Mintel (2007) reported that the gluten-free foods and beverages market in 2006 was $ 700 million and is projected to grow annually at a rate of 15-25% to $ 1.3 billion by 2010. The largest numbers of new introductions were in bakery goods and snacks, which were 22.70% and 17.5%, respectively, of the 1,339 gluten-free products introduced in 2006.

### 2.2.3   Creating gluten-free foods is challenging

Unfortunately, the quality of gluten-free products has not kept up with the rising demand. Gluten replacement in food presents several challenges. First, because gluten is a unique structure-building protein, its removal from baked products (especially bread) and pasta, results in very poor sensory qualities and product shelf-life (Gallagher and others 2004). For example, gluten-free bread is typically crumbly and does not hold together, making it difficult to prepare a sandwich. Second, gluten-free products are made primarily from isolated starches, thus are poor in fiber, protein, vitamins and minerals (Engleson and Atwell 2008; Berti and others 2004). Third, gluten-free products are more expensive than comparable conventional products because of the added burden of ensuring the absence of cross-contamination and because production is at a smaller scale (Mintel 2007). Generally, gluten-free products are 240% more expensive than their gluten-free counterparts (Lee and others 2007). Fourth, there is limited availability of gluten-free selections in dining establishments (Mintel 2007), regular grocery stores (Lee and others 2007), and schools (Swientek 2008). Although studies have been conducted to address some of these technological and nutritional issues, there is still a need to develop gluten-free ingredients and processes that are safe and economical for celiacs and those with gluten sensitivities.

### 2.2.4 Sorghum is a safe ingredient for gluten-free products

Sorghum is a cereal grain that is safe for celiac patients (Ciacci and others 2007). Sorghum flour is an attractive alternative to wheat flour for the celiac market because of its neutral flavor and the use of hybrids with a white pericarp. These white grained sorghum lines produce flour similar to wheat flour in appearance and do not impart an unusual color to the flour. Furthermore, sorghum utilization helps address food security issues because it is a drought resistant crop that easily withstands harsh cultivating conditions in impoverished regions of Asia and Africa. The United States is the world's top sorghum producer, followed by India and Nigeria (U.S. Grains Council 2008). In 2007, the U.S. produced 12.83 million metric tons, or 20% of the world's sorghum supply. Sorghum is the third most widely produced crop in the U.S. and fifth in the world. Although mostly supplied to the feed industry in the U.S., sorghum is an important staple in parts of Asia and Africa (Dendy, 1995). Thus, sorghum is important economically and it ensures food security in a number of countries worldwide.

### 2.2.5 Cooking sorghum reduces its nutritional value

In Asia and Africa, sorghum is traditionally prepared in a number of ways including porridges, flat breads, alcoholic beverages, and snacks (Murty and Kumar 1995). However, cooking sorghum, especially wet cooking, reduces its digestibility (Emmambux and Taylor 2009; Ezeogu and others 2008; Nunes and others 2004; Duodu and others 2003; Duodu and others 2002; Zhang and Hamaker 1998; Hamaker and others 1986), making it less available for the body to use. For celiac patients who typically suffer from malnutrition due to poor nutrient absorption, it is even more important for nutrients to be more readily available. Thus, a challenge exists to make sorghum proteins more digestible. Additionally, in order to make sorghum protein a commercially viable ingredient, it has to be concentrated and/ or isolated at an industrial scale using processes and/ or chemicals that are compatible with food grade applications.

In this review, we describe methods used to concentrate, isolate, and modify sorghum proteins and also identify new developments in uses of sorghum and sorghum proteins in gluten-free foods, specifically in staples like bread and pasta.

## 2.3 OVERVIEW OF SORGHUM PROTEINS

As extensive literature is available on characterizing sorghum proteins, only an overview is given to serve as basis for discussion. For a recent review on sorghum protein chemistry and structure, see Belton and others (2006).

### 2.3.1 Sorghum Proteins

Sorghum (*Sorghum bicolor* L. Moench) grain has protein content varying from 6 to 18%, with an average of 11% (Lasztity 1996). Sorghum proteins can be broadly classified into prolamin and non-prolamin proteins. Kafirins, the major storage proteins, are classified as prolamins, and as such, they contain high levels of proline and glutamine and are soluble in non-polar solvents such as aqueous alcohols (Shewry and Tatham 1990). Kafirins account for 77 to 82% of the protein in the endosperm, whereas non-prolamin proteins (namely, albumins, globulins, and glutelins) make up about 30% of the proteins (Belton and others 2006). Since maize and sorghum are closely related genetically (both belong to the same tribe of the grasses (Andropogoneae), the large volume of research on maize prolamins, called zein, has served as a framework for studying kafirins. Shull and others (1992) even utilized procedures developed for maize to characterize the proteins of sorghum based on solubility, molecular weight, and structure. This review primarily focuses on kafirin proteins.

### 2.3.2 Kafirin classification and microstructure

Kafirins are classified as either α, β, γ, or δ based on molecular weight and solubility. Depending on whether it is floury or vitreous, sorghum endosperm contains about 66-84% α-kafirin, 8-13% β-kafirin and 9-21% γ-kafirin and low levels of a poorly characterized δ-kafirins (Belton and others 2006; Lasztity 1996). The α-kafirins are divided into two groups of polypeptides with molecular weights ($M_w$) of 23 and 25 kDa. These proteins are rich in non-polar amino acids and are found primarily as monomers and oligomers. These proteins do not crosslink extensively and form mainly intramolecular disulfide bonds. The β-kafirins have a $M_w$ of ~18 kDa, are rich in the sulphur-containing amino acids methionine and cysteine, and are found in monomeric and polymeric forms. The γ-kafirins have a $M_w$ of approximately 20 kDa and are rich in the amino acids proline, cysteine and histidine. These subunits are found as oligomers and polymers. Both β- and γ-kafirins form intermolecular and intramolecular disulfide bonds and are highly crosslinked. The δ-kafirins have a $M_w$ of about 13 kDa and are

rich in methionine (Belton and others 2006). Overall, sorghum prolamins are rich in glutamic acid and non-polar amino acids (proline, leucine and alanine), but almost absent with the essential amino acid lysine.

The microstructure of kafirins in relation to the glutelin protein matrix and starch granules, as well as its reaction to chemicals and processing, have been studied using scanning and transmission electron microscopy (Elkhalifa and others 2006; Duodu and others 2002; Oria and others 2000; Oria and others 1995a; Shull and others 1992; Rooney and Pflugfleder 1986; Taylor and others 1984a; Hoseney and others 1981; Seckinger and Wolf 1973), as well as confocal laser scanning microscopy (Choi and others 2008; Schober and others 2007; Wu and others 2007). The above studies have shown that kafirins are located primarily in spherical protein bodies, which are embedded in a glutelin protein matrix, and are surrounded by starch granules. A schematic representation of this relationship is shown in Fig. 2.1 and Fig. 2.2. The protein bodies are 0.4 to 2μm in diameter (Taylor and others 1984a), with an outer "shell" composed mainly of crosslinked β- and γ-kafirins, and an interior comprised predominantly of α-kafirin (Duodu and others 2003; Shull and others 1992).

While kafirins in most sorghum cultivars are tightly bound in spherical protein bodies, sorghum protein bodies in the highly digestible mutant sorghum cultivar P851171 grown at the Purdue University Agronomy Research Center were irregularly shaped with numerous invaginations (Oria and others 2000). These authors attributed the ease of digestibility to this unique microstructure. The invaginations provided a greater surface area for enzymatic digestion; the highly digestible α-kafirins were more homogenously dispersed throughout the interior of the protein body rather than simply localized in the central portion; and, the poorly digestible γ-kafirins were concentrated at the base of the invaginations of the protein body rather than at the protein body periphery encapsulating α-kafirins like in normal sorghum cultivars.

### 2.3.3  Sorghum protein digestibility

Duodu and others (2003) extensively reviewed the factors affecting sorghum protein digestibility, which were broadly categorized as exogenous and endogenous factors. The former involves interactions of proteins with non-protein components (e.g., polyphenols, phytates, lipids, starch and cell wall components), while the latter entails only protein-protein interactions.

Polyphenols, lipids and cell wall components form complexes with kafirins that are resistant to digestion. Cooking enhances the interaction of kafirins with these compounds, further reducing protein digestibility (Duodu and others 2003). A 50% reduction was observed as a result of the complexation of total kafirins with sorghum condensed tannins (Taylor and others 2007). Taylor and others (2007) also found that sorghum tannins bound preferentially to γ-kafirins than to either α- and β-kafirins because of the high proline content of γ-kafirins. However, not all sorghum lines contain tannins, thus this would only be an issue in sorghum lines containing tannin. Likewise, phytic acid also complexes with kafirins. Unlike the previously mentioned components, however, phytic acid content is reduced upon cooking, reducing its role in protein digestibility. Starch affects sorghum protein digestibility differently. Although the bulk of the literature suggests that sorghum proteins inhibit starch gelatinization and its digestion (Ezeogu and others 2008; Duodu and others 2002), the presence of starch mutually reduces sorghum protein digestibility (Wong and others 2009; Duodu and others 2003). *In vitro* protein digestibility, however, can be improved with the addition of α-amylase to either raw (Wong and others 2009) or cooked (Duodu and others 2002) sorghum flour. Likewise, the presence of the glutelin matrix that binds kafirin protein bodies and starch granules reduces protein digestibility (Wong and others 2009).

In general, kafirins tend to be more hydrophobic than other cereal prolamins (Belton and others 2006). While these proteins have some hydrophilic tendencies in the raw state, cooking at high moisture emphasizes their hydrophobicity. This reversal in water absorption upon cooking may be due to extensive disulfide bonding, which results in the polymerization of kafirin monomers and realignment of kafirin into β-sheets. This structural change then prevents swelling, imbibition of water, and reduces protein's susceptibility to proteolysis (Emmambux and Taylor 2009; Belton and others 2006). Cooked sorghum showed a decrease in the amount of albumin, globulin and kafirins and a concomitant rise in the percentages of cross-linked glutelin and nonextractable proteins (Hamaker and others 1986). Additionally, cooking sorghum in high moisture resulted in an increase in the amount of pepsin-indigestible proteins from 19.3% (raw) to 35.2% (cooked). These results presented by Hamaker and others (1986) indicated that the reduction in protein digestibility during cooking at high moisture was brought about by protein polymerization. On the other hand, protein digestibility of sorghum cooked in limited water, such as popping or extrusion, was either greater than (Hamaker and others 1994; Fapojuwo and

others 1987; Mertz and others 1984; MacLean and others 1983), or the same as that of uncooked sorghum (Parker and others 1999; Dahlin and Lorenz 1993). This phenomenon was attributed by Parker and others (1999) to the explosive disruption of the cell walls and expansion of starch, leading to immediate accessibility of enzymes to proteins.

### 2.3.4 Kafirin functionality in food systems

Protein functionality is related to protein size, molecular structure and conformation, charge distribution, and molecular interactions. The functional roles of proteins in food systems include solubility, viscosity, water binding, gelation, elasticity, emulsification, foaming, gas holding capacity, and fat and flavor binding, and these properties define their applications in foods. Extensive literature on these properties exists and a discussion of which is beyond the scope of this review (see for example Sikorski 2001; Damodaran and Paraf 1997; Zayas 1997; Kinsella and Soucie 1989; Nakai 1983). This section focuses on the functionality of kafirins.

Oom and others (2008) studied the rheological properties of kafirins in a viscoelastic dough system. Their study showed that although the extensional viscosity of isolated kafirin dough immediately after mixing was similar to those found in gluten based dough, it became rapidly stiff over time. Oom and others (2008) speculated that this was due to disulfide crosslinking of kafirin monomers. When kafirin was mixed with starch and water, however, no dough could be formed. The authors inferred that kafirin's inability to form composite viscoelastic doughs could be a result of its extremely hydrophobic nature (i.e., its exclusion of water prevented its hydration and plasticization).

Kulamarva and others (2004) found that sorghum flour has poor viscoelastic properties and that the observed rheological properties result primarily from starch gelatinization. These authors studied the effects of water level (70, 80, 90 and 100% flour weight basis) and temperature of mixing ($22^{o}$C and $100^{o}$C) on the rheological properties of sorghum flour using the Instron Universal Testing Machine. Extensibility was greater at higher water levels. Dough mixed with boiling water had increased extensibility, reduced hardness, increased cohesiveness, and higher gumminess values, which were most likely due to starch gelatinization. In the same work, they conducted parallel plate oscillatory tests with a dynamic rheometer. Dough samples were subjected to a constant stress of 6 Pa with frequency ranging from 0.1 to 100 Hz at $25^{o}$C.

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The storage modulus was reduced with increasing water levels because of dilution. And due to starch gelatinization, it was also lower in dough mixed with boiling water than with cold water.

Schober and others (2007) studied the rheological properties of sorghum dough subjected to sourdough fermentation and investigated the effect of adding hydroxypropyl methyl cellulose (HPMC) to the dough. Dynamic oscillatory testing at 1 Hz in the linear viscoelastic region was conducted by using a serrated plate measuring system with the following temperature profile to simulate the baking and cooling processes: (1) a linear temperature ramp from 25 to 95$^{o}$C in 47 min; (2) 10 min at 95$^{o}$C; and, (3) a linear gradient down from 95 to 25$^{o}$C in 47 min. The target strain was 5 x 10$^{-4}$. Compared with maize and potato starch doughs, sorghum flour dough had a higher /G$^{*}$/ (absolute value of the complex dynamic shear modulus) over the temperature range tested and lower phase angles, indicating it was firmer or more resistant to deformation and more elastic, respectively. Additionally, unlike in starch doughs, these parameters changed over a broader range for sorghum flour doughs due to its broader particle size distribution and because of delayed starch gelatinization resulting from sorghum starch particles being embedded in the surrounding protein. Sorghum dough became thinner as a result of sourdough fermentation. There was no notable difference in /G$^{*}$/ seen between sorghum flour dough with and without sourdough treatment. After gelatinization, however, /G$^{*}$/ was significantly higher for sorghum flour dough with sourdough treatment. Hence, sourdough treatment resulted in a stronger starch gel upon subsequent heating.

In another study, Schober and others (2005) utilized a texture analyzer equipped with a forward extrusion cell having a 10 mm nozzle to analyze bread batters using flours from different sorghum hybrids. The maximum extrusion force (at 8 to 18 mm distance) indicated batter firmness/ consistency. Batter consistency varied amongst the samples, with extrusion forces ranging from 3.5 to 10.1 N. This test was also used to adjust the amount of water added in order to obtain constant consistency of 4.9 N for bread baking as a method to standardize the amount of water added to the bread formula. Bread made from commercial flour with this consistency was of good quality, hence was taken as the standard. The authors found that low consistency batters (5% more water than the standard water content of 105%, flour weight basis) had improved specific volume and crumb texture.

The effect of fermentation on the functional properties of sorghum flour was also described by Elkhalifa and others (2005). These authors found that the protein solubility of

sorghum flour increased in the acidic range (pH 2-4); oil-binding capacity, emulsifying capacity, and emulsifying stability increased;   water-binding capacity decreased; and,   no foaming capacity was observed in either fermented or unfermented flours.   However, since this study considered sorghum flour in its totality and did not focus on either sorghum proteins or kafirins, it is possible that other factors, aside from protein modification resulting from fermentation, could have possibly played a role in the observed changes in functionality.

## 2.4   FOOD USES OF SORGUM-BASED INGREDIENTS

Gluten-free bread and pasta that have the same quality of wheat-based counterparts are the most highly desired foods by celiacs, and yet are the most difficult to formulate.   A plethora of reviews and articles on gluten-free foods, including sorghum breads and pasta, exist.   Recent publications on these include those by Schober and others (2006), Taylor and others (2006) and Gallagher and others (2004), and books edited by Gallager (2009) and by Arendt and Dal Bello (2008), to which the reader is referred.   This review describes recent developments in the uses of kafirins in bread and pasta.

Hamaker and others (2008) patented the production of leavened products made from non-wheat cereal proteins.   The inventors claim that the composition comprises of non-wheat starch, flour, or a mixture of non-wheat cereal storage proteins from either maize, sorghum, millet, rice or oat and a co-protein such as casein, elastin, γ-zein or γ-kafirin.   The inventors explained that co-proteins, when mixed with cereal prolamins (especially zein and kafirin), will stabilize the β-sheet formation in the non-wheat prolamin and facilitate the formation of dough that retains its viscoelasticity for an extended period of time under room temperature.   Stabilization of the β-sheet conformation is believed to be brought about by binding of the prolamin and co-protein. An example of the method for making bread is: (a) conditioning a mixture of the prolamin and co-protein between 35 and 50$^{o}$C with 5 to 25% (w/w) moisture content for 1 to36 h, preferably between 12 and 24 h; (b) preparing a leavened dough with the conditioned protein mixture, starch, water, sugar, salt, ammonia and dry yeast in a mixer at 35$^{o}$C; (c) proofing the dough for 35 min at 35$^{o}$C; and, (d) baking at 220$^{o}$C for 20 min.   Conditioning the protein mixture is thought to meld the prolamin and co-protein to form a network comparable to wheat gluten.   With the exception of zein, which needs to have a moisture content during conditioning between 10 and 25% (w/w), the moisture content of the prolamin/ storage protein or co-protein is sufficient to

hydrate the proteins and convert it from the glassy to flowable state. It is worth noting that mixing the leavened dough is done at a higher temperature than most traditional processes, which are usually done at ambient temperature. This is probably because proteins like zein require higher temperatures to exhibit extensibility.

A patent by Engleson and others (2009) describes a system for gluten replacement of food products, including yeast-leavened dough, involving the use of a combination of a gluten-free gas-retaining agent and setting agent. The cited gas-retaining ingredients include polymers like chewing gum base, butyl rubber, paraffin, and petroleum wax, to name a few; and, setting agents include kafirin, zein, egg, whey and soy proteins, caroubin, casein, shellac, and hydrocolloids, to name a few. The gas-retaining agents enable the dough to hold carbon dioxide generated by the leavening agent within the gas cells, while the setting agents bring about strain hardening upon increase in temperature and evaporation of water. The patent describes prepraration of bread dough at room temperature using a kitchen bowl mixer, proofed at $46^{\circ}C$ with a relative humidity of 85%, and then baked for 30 min at $221^{\circ}C$. Leavened pan breads were reported to have specific volumes of 3.8 to 6.0 mL/g, similar to those of wheat bread. The patent claims that this gluten replacement system can be used to make pasta, crackers, pizza crust, and leavened bread.

Suhendro and others (2000) studied the effects of the modes of cooking and drying on the qualities of noodles made from decorticated sorghum flour. These cooked a mixture of 100g sorghum flour, 90 mL water and 1% salt using either a hot-plate or a microwave oven. The preheated mixtures were passed through a forming extruder to produce the noodles. Three methods of drying were evaluated (slow air drying, one-stage hot air drying, and 2-stage drying with high and low humidity). Noodles preheated in the microwave yielded better qualities (i.e., firmer, less chewy, less sticky, and low dry matter losses) because less starch gelatinization occurred when the noodles were heated in the microwave rather than when they were cooked on a hot plate. The 2-stage drying method yielded the best noodles because hot, moist drying enhanced amylose mobility and reassociation. Starch retrogradation hinders water absorption, thereby reducing the amount of starch leaching into the cooking water. Rapid hot air drying shortened the period for starch retrogradation, and the temperature for slow air drying was too low to promote amylose mobility; thus, both methods resulted in inferior noodle quality. Timing of amylose solubilization and dispersion, noodle formation and amylose retrogradation were

critical in obtaining noodles of good quality.  These authors also reported that finer flour yielded better noodles.

In studying noodles made using four sorghum grain varieties, Liu (2009) found that sorghum noodle quality was also highly dependent on starch properties.  Liu (2009) prepared noodles with a formulation containing sorghum flour, corn starch, dried egg whites, whole eggs, xanthan gum, salt and water.  The ingredients were blended in a batch mixer, kneaded by hand, sheeted and cut using a noodle machine, and then cooked in boiling water.  Textural properties were evaluated with a texture analyzer and starch pasting properties were analyzed with a rapid visco-analyzer (RVA).    Sorghum flours with lower starch pasting peak viscosity, shorter peak development time and lower peak temperature produced more desirably firm noodles.  While shorter peak development time and low gelatinization temperature (indicators of rapid starch swelling and gelatinization) are also desired in making wheat flour noodles, contrary to sorghum flour noodle, high starch pasting peak viscosity (a measure of starch swelling power) is more desirable.  However, Liu (2009) did not find starch pasting properties to be significantly related to cooking loss.  Similar to the findings reported by Suhendro and others (2000). Liu (2009) reported a positive correlation between amylose content and cooked noodle firmness.  Unlike in wheat noodles where high protein content of the flours results in better noodles, Liu (2009) found that sorghum protein content was not related to cooked noodle firmness or tensile strength.

Pre-cooked pasta based on sorghum flour has also been prepared by extrusion cooking and forming (Cheng and others 2007). This pasta had similar cooking quality (water absorption and cooking loss) as commercial wheat-based pasta.  Although non-wheat noodles, including those made from sorghum flour, were reported to rely primarily on starch for their quality (Liu 2009; Suhendro and others 2000), the reasonably good quality of pre-cooked sorghum pasta observed by Cheng and others (2007) can also possibly be attributed to modification of sorghum proteins during extrusion.  Similar to the disruption of maize protein bodies and dispersion of α-zein during extrusion (Batterman-Azcona and others 1999), the relatively high mechanical energy input in the pre-cooked pasta extrusion process may have led to the disruption of kafirin protein bodies and its formation of a protein structural network which reduced dry matter losses. This was however not confirmed experimentally, and more research is needed in this area.  In wheat flour pasta, proteins are responsible for ensuring strength and quality of the products, and improving the functionality of sorghum proteins could lead to improved sorghum pasta quality.

To summarize this section, sorghum is safe for celiacs and individuals with gluten sensitivities, and improving its protein digestibility is of exceeding importance. Better protein digestibility not only increases sorghum utilization in foods and offers gluten-intolerant individuals an alternative nutritional source but also helps populations in developing nations, for which sorghum is a diet staple, to maximize protein intake. And, although protein digestibility is the foremost concern, improved protein functionality further expands its application in foods. Developing a concentrated source of sorghum proteins with enhanced nutritional and functional characteristics can open more doors for utilization of sorghum in foods. Consequently, this review next presents methods for extraction and concentration of sorghum proteins and describes strategies for protein modification.

## 2.5 ISOLATION OF SORGHUM PROTEINS

### 2.5.1 *Wet-milling of sorghum*

Wet-milling is a physico-chemical separation of the components of grain, namely, germ, bran, fiber, starch, and protein. Corn is the grain traditionally used for wet-milling but its shortage during World War II led to the utilization of sorghum grain as the starting raw material in the commercial production of starch and dextrose (Zipf and others 1950). For 22 years, sorghum was used in a commercial wet-milling facility in Corpus Christi, Texas (Rooney and Serna-Saldivar 2000), but its use was later discontinued because of incomplete starch recovery, low oil yield, and high wax content in the grain (Yang and Seib 1995). Additionally, the economic competitiveness of using sorghum over corn was gone because the price of grain sorghum rose and almost paralleled that of corn. To date, there are no known commercial sorghum wet-milling operations in the U.S. Munck (1995) describes an elaborate wet-milling process with ten possible products (germ, crude oil, refined oil, fiber, protein, protein meal (referred to as sorghum gluten meal), wet starch, dry starch, dextrin, and glucose) while Rooney and Serna-Saldivar (2000) illustrate a commercial wet-milling process for grain sorghum. A schematic diagram of a simplified wet-milling process is shown in Fig. 2.3.

Given the intricate attachment of sorghum proteins and starch, it is not surprising that poor starch recovery and residual protein content in starch are of great concern in sorghum wet-milling. Using the same wet-milling process, starch recovery and residual protein content in starch from yellow maize is about 90% and 0.12%, respectively, while that from regular

sorghum is only about 86% and 0.20%, respectively (Perez-Carillo and Serna-Saldivar 2006). Of interest to this review on sorghum proteins is the amount of protein recovered and the use of chemicals and enzymes in wet-milling that facilitate the separation of sorghum grain components. Sorghum protein fraction (also referred to as gluten fraction) yields (i.e., the dry weight of the protein fraction obtained from wet-milling divided by the initial dry total solids weight in the kernel multiplied by 100) ranging from 8.23% to 25.60% have been reported (Xie and Seib 2000; Wang and others 2000; Buffo and others 1998; Moheno-Perez and others 1997). Protein contents of the sorghum protein (gluten) fraction range from 44.31% to 58.20% (Xie and Seib 2000; Buffo and others 1998).

Critical to the wet-milling process and the subject of most sorghum wet-milling studies is steeping of sorghum. Sorghum grain is steeped in water to toughen the bran and soften the endosperm for easy separation. Chemicals and enzymes can be added to the steeping water to facilitate the separation of grain components and increase starch recovery. Sulfur dioxide ($SO_2$), sodium metabisulfite, sodium bisulfite or sodium hydrogen sulfite, with an effective concentration of 0.05 to 0.30% $SO_2$, are typically added to solubilize the protein matrix enveloping the starch granules in the endosperm (Perez-Carrillo and Serna-Saldivar 2006; Serna-Saldivar and Mezo-Villanueva 2003; Xie and Seib 2000, 2002; Wang and others 2000; Buffo and others 1998; Moheno-Perez and others 1997; Yang and Seib 1995,1996; Zipf and others 1950). Sometimes, lactic acid (0.40 to 1.4% (w/w) is also added to facilitate protein solubilization. Cell-wall-degrading enzymes and proteases have also been used in wet-milling of sorghum in attempt to increase starch yield and reduce protein content in the starch (Perez-Carrillo and Serna-Saldivar 2006; Serna-Saldivar and Mezo-Villanueva 2003; Wang and others 2000; Moheno-Perez and others 1997). While the addition of protease significantly increased starch recovery (Perez-Carrillo and Serna-Saldivar 2006; Mezo-Villanueva and Serna-Saldivar 2004), the use of cell-wall-degrading enzymes alone did not have a significant benefit (Perez-Carrillo and Serna-Saldivar 2006; Wang and others 2000; Moheno-Perez and others 1997). These enzymatic studies are additional evidences showing the complexity of starch-protein binding in sorghum.

Steeping experiments have been done on temperature and holding time, and the optimum conditions lie within 48 to 55 °C for 24 to 48 h. Grain to steep water ratio in laboratory wet-milling is usually 1:2 (Perez-Carrillo and Serna-Saldivar 2006; Serna-Saldivar and Mezo-

Villanueva 2003; Xie and Seib 2000, 2002; Wang and others 2000; Moheno-Perez and others 1997) whereas that in commercial wet-milling is 1:5 (Rooney and Serna-Saldivar 2000).  Whole grain sorghum is the typical starting material.  Using decorticated grain as the starting raw material is not beneficial due to high starch losses (Yang and Seib 1996; Zipf and others 1950), lack of improvement in starch brightness and insignificant reduction in protein contamination of starch (Yang and Seib 1996).  The use of sorghum grits as the starting raw material, instead of whole grain sorghum, yielded starch with less protein contamination and improved brightness (Higiro and others 2003).

### 2.5.2  *Traditional methods of protein extraction*

Sorghum proteins traditionally have been extracted and classified based on the Osborne procedure (Osborne 1907).  This classification method divides proteins into water soluble albumins, salt soluble globulins, alcohol soluble prolamins, and acid or base soluble glutelins (Wrigley and Bekes 2001; Hamaker and others 1995; Taylor and others 1984b; Virupaksha and Sastry 1968).  This method, however, does not cleanly separate protein fractions and generally results in significant overlap among the fractions.  Many variants of this method have been used to extract sorghum proteins.  The Landry-Moureaux method later further divided prolamins into those extractable in aqueous alcohol alone and those extractable in aqueous alcohol plus a reducing agent (reviewed by Hamaker and others 1995).  In this procedure, sequential extraction results in the following protein fractions:  albumins and globulins extracted with NaCl solution (fraction I); kafirin-1 extracted with 60% t-butanol (fraction II); kafirin-2 (also referred to as crosslinked kafirin) extracted with 60% t-butanol with 2-mercaptoethanol (2-ME) (fraction III); glutelin-like proteins extracted with alkali borate buffer with 2-ME (fraction IV); true glutelins extracted with alkali borate and sodium dodecyl sulfate (SDS) (fraction V); and, nonextractable proteins determined by protein content analysis of the residue.   Although the Landry-Moureaux method fine-tuned the Osborne procedure, it still does not provide much information about the functionality of sorghum proteins.

### 2.5.3  *Alkaline extraction*

Wu (1978) obtained sorghum proteins from whole ground sorghum by using an alkaline extraction process.  Extraction was carried out by preparing a slurry with 150 g of ground sorghum and 900 mL 0.1-0.15 N sodium hydroxide solution, pH 11.8-11.9.  The slurry was

centrifuged and the supernatant was collected and adjusted to pH 4.8 to precipitate the proteins. The protein concentrate in the form of the precipitate was freeze-dried. The concentrates had protein contents ranging from 48 to 60%, depending on grain variety. Solubilities of the concentrates were 90% at a pH range of 8.7 to 10.8; 15 to 22% at pH 2.1; and, were insoluble between pH 3.5 to 5.8. The authors of this study did not identify the types of protein extracted. However, judging from the amount of protein extracted, some kafirin may have been solubilized by this process. Assuming the bulk of the protein was albumin/globulin and glutelins, some 5-15% of the extracted protein may have been kafirin. In addition, it was not noted if any modification occurred to the proteins due to the extreme pH used in the extraction process.

### 2.5.4  *Separation of non-prolamins and prolamins*

Hamaker and others (1995) used a procedure first applied to extract maize proteins that differentiated non-prolamins (or non-kafirins) from prolamins (or kafirins). In this method, flour samples were first extracted with sodium chloride to remove the albumins, globulins and nonprotein nitrogen contained in the supernatant. The resulting pellets were then extracted with sodium borate, a detergent (SDS), and 2-ME at pH 10.0, with a flour-solvent ratio of 1:10. After a 1-h extraction, the suspension was centrifuged, and then 60% t-butanol was added to the supernatant to precipitate the detergent-extractable nonkafirins. After standing for 2 h with occasional stirring, the mixture was centrifuged, and then the supernatant, containing kafirins, was separated from the pellet. This procedure allowed kafirins to be obtained as one group and facilitated further identification of the different types of kafirins. However, because these proteins were extracted primarily for characterization purposes, and not for food use, selecting food-grade chemical reagents was of little concern.

Following the abovementioned procedure by Hamaker and others (1995), Park and Bean (2003) investigated the factors affecting sorghum protein extraction and then optimized these conditions to reduce extraction time. Their studies revealed that pH, detergent type, reducing agent type and sample-to-solvent ratio significantly affected protein extraction. From pH 2.5 to 10, the amount of protein extracted by SDS increased with increasing pH. SDS, an anionic detergent, was exceedingly superior to the cationic detergent dodecylammonium bromide and zwitterionic detergent SB 3-12, regardless of concentration. SDS concentration of 2% extracted the most amount of protein, with no further increases in extraction at higher concentrations. β-

ME at 2% extracted more proteins than either dithiothreitol or tris(2-carboxyethyl) phophine hydrochloride.  In contrast to the 1:10 flour-solvent ratio used by Hamaker and others (1995), Park and Bean (2003) found that the optimum ratio was 1:20.  Furthermore, by pooling the protein extracts from three 5-min extractions, Park and Bean (2003) shortened total extraction time from 1 h to 21 min, while obtaining the same amount of protein.    They also determined that the same non-kafirins were precipitated by 60% t-butanol, 60% 1-propanol, and 70% ethanol.  Thus, these solvents can be interchanged.  Additionally, acetone can be used to precipitate kafirins.

### 2.5.5  Use of sonication

Sonication has been utilized to improve extraction of sorghum proteins and to rapidly separate sorghum protein and starch (as in the case of sorghum starch isolation).  Bean and others (2006) investigated the effects of various extraction and precipitation conditions, including the use of ultrasound, on recovery and purity of kafirins.  These authors extracted protein from whole ground sorghum flour with 70% ethanol at 50$^{\circ}$C for 1 h, with and without reducing agents (sodium metabisulfite, glutathione and cysteine), and with 4 min sonication. Lipid was first removed from the extract by diluting ethanol to 60% and centrifuging.  The supernatant was then collected for protein precipitation.  Protein sedimentation was done by further diluting the ethanol solution from 50 to 30% with water, with or without sodium chloride and with or without lowering the pH to 2.5.  After continual mixing and centrifugation, the precipitates were collected and air-dried overnight at room temperature, and then analyzed for protein content and characterized.  The authors found that extracting with ethanol alone resulted in poor protein purity (31-52% protein content), and that the addition of a sonication step increased protein content by 15 to 26%.  The use of ethanol with either glutathione or sodium metabisulfite (without sonication), on the other hand, yielded a larger percentage of extracted protein (about 70-80%).  These reducing agents were preferred over β-ME due to their suitability for foods.  Lowering pH enhanced protein precipitation because kafirins have low levels of the positively charged amino acids arginine, lysine and histidine, which are responsible for the solubility of proteins at low pH.  While the addition of NaCl increased the amount of protein precipitated in some conditions, overall, its addition did not show a significant improvement in the amount of protein precipitated over the other methods (i.e., either lowering ethanol

concentration or reducing pH). Precipitating sorghum proteins by dilution to 50% ethanol, with or without NaCl and lowering pH to 2.5, yielded the highest protein content (purest precipitate) under most extraction conditions.

Zhao and others (2008) also used sonication to extract proteins from sorghum and characterized these using size exclusion and reversed-phase high performance liquid chromatography. A sorghum meal slurry containing sodium borate (pH 10.0) and SDS was sonicated at 10W for 30 s. Sonication is believed to reduce the molecular weight of large proteins by breaking covalent bonds through shear degradation. While sonication extracted more polymeric proteins than SDS borate buffer alone, the amount of proteins this method extracted was fewer than that extracted by a 24-h extraction with SDS borate buffer.

Park and others (2006) also used sonication to disrupt sorghum protein structures in order to isolate sorghum starch. Sorghum flour was mixed with various protein extraction buffers containing sodium borate buffer, SDS and different reducing agents (β-ME, dithiothreitol, and sodium metabisulfite), and then sonicated. The authors concluded that 2-min sonication of sorghum flour with 12.5 mM sodium borate buffer, pH 10, with 0.5% SDS (w/v) and 0.5% sodium metabisulfite (w/v) were the optimum conditions for producing sorghum starches with protein contents below 0.06%.

### 2.5.6 *Extraction of sorghum polymeric proteins*

Differential solubility is often used in studying the molecular weight distribution of wheat proteins. In wheat, this differentiation is useful in determining the strength of the dough and, therefore, is a predictor of functionality. Differentiating sorghum proteins on the basis of solubility provides insight into the extent of crosslinking. Several authors have utilized this technique to study sorghum proteins (e.g. Ioerger and others 2007; Nunes and others 2005; El Nour and others 1998; Oria and others 1995a,b;) . These studies have shown that high molecular weight polymeric proteins were linked by disulfide (SS) bonds and that these polymers were primarily made up of γ-kafirins. The α- and β-kafirins were found as monomers and also participated in the formation of oligomers. Vitreous endosperm had higher amounts of crosslinked proteins than did floury endosperm. Formation of high molecular weight aggregates was promoted by cooking. While most of these authors utilized the previously described procedures for extracting kafirins, Ioerger and others (2007) used a different method for

extracting sorghum proteins based on solubility. In their study, a multistep extraction procedure divided sorghum proteins into soluble proteins (SP), insoluble proteins (IP) and residue proteins (RP). SP were those proteins extracted from sorghum flour with sodium borate, pH 10.0, buffer with 2% SDS. After continuous shaking and centrifugation of this mixture, IP were extracted from the pellet with sodium borate, pH 10.0, buffer using sonication (30 s at 10 W). Then, after centrifugation, RP were extracted from the remaining pellet with sodium borate, pH 10.0, buffer with 2% SDS and 2% β-ME. After centrifugation, the protein content remaining in the pellet was analyzed. Aliquots of the extracts were analyzed by size exclusion-high performance liquid chromatography (SEC) and the percentages of each extract were determined. Floury endosperm had a higher SP percentage (47.2%) than vitreous endosperm (36.7%), while the RP portions did not differ significantly. A more notable difference was seen in the IP portion wherein vitreous endosperm had a greater proportion of IP (45.3%) than floury endosperm (35.9%). Furthermore, the IP fraction of the vitreous endosperm had more polymeric proteins than the IP fraction of the floury endosperm. These factors indicated that proteins in vitreous endosperm were more extensively crosslinked and had higher molecular weights than proteins in floury endosperm. Ioerger and others (2007) postulated that the SP and IP extracts were analogous to the kafirin-1 (or fraction II) and kafirin-2 (or fraction III) fractions, respectively, of the Landry Moureaux procedure; and, that the RP fraction was most likely made up of non-prolamin proteins.

### 2.5.7  *Glacial acetic acid extraction*

Taylor and others (2005) developed a kafirin extraction method using glacial acetic acid because existing procedures pose problems for the food industry. For instance, they noted that t-butanol is toxic and that aqueous ethanol is not acceptable to certain religions. The authors hypothesized that the low dielectric constant of glacial acetic acid (6.1) enables it to dissolve highly hydrophobic proteins such as kafirin. Dielectric constant of a solvent is inversely proportional to the extent of interaction occurring between two charged particles in solution. As glacial acetic acid has a low dielectric constant, proteins tend to unfold and hydrophobic groups interact with the solvent just as easily as these would with each other. In this study, aqueous alcohol extractants (70% ethanol at 70$^{o}$C and 55% isopropanol at 40$^{o}$C), each containing sodium metabisulfite and sodium hydroxide, were compared against extractants containing glacial acetic acid with and without sodium metabisulfite at 25$^{o}$C. Additionally, the authors tested the effect of

presoaking sorghum flour in sodium metabisulfite prior to extaction with glacial acetic acid at 25$^o$C. After extraction, kafirin preparations were defatted with hexane. The results of their experiments showed that after defatting, the purity of the kafirins extracted with either aqueous alcohol extracts were not significantly different from the purity of the kafirins obtained by glacial acetic acid extraction with presoaking in 0.5% sodium metabisulfite for 16 h. Pretreatment with sodium metabisulfite was necessary in obtaining the desired purity as extraction with glacial acetic acid alone had poor kafirin yield and purity.

Wang and others (2009) compared the properties of kafirins isolated from sorghum dried distiller's grain with solubles (DDGS) using the acetic acid method developed by Taylor and others (2005), an acidic-ethanol method originally used for maize, and the alkaline-ethanol method modified for sorghum by Emmambux and Taylor (2003). In the acetic acid method, sorghum was presoaked in sodium metabisulfite for 16 h prior to extraction with glacial acetic acid. The resulting protein was defatted with petroleum ether. In the acidic-ethanol method, defatted sorghum DDGS was mixed with 70% ethanol, the pH was adjusted to 2.0 using HCl, and then sodium sulfite was added. After continual stirring for 2 h at 78$^o$C, the mixture was centrifuged and the supernatant was collected and dehydrated by a rotary evaporator. A second defatting procedure was performed. In the alkaline-ethanol method, sorghum DDGS was mixed with 70% ethanol, 0.35% NaOH and 0.5% sodium metabisulfite, and then stirred for 1 h at 70$^o$C. Next, the mixture was centrifuged, then the supernatant was diluted with distilled water to 40% ethanol. This suspension was held at -20$^o$C overnight to promote precipitation, and then centrifuged. The pellet was rinsed with distilled water, dried at 49$^o$C overnight, and then defatted. Analysis of protein content showed that acetic acid and alkaline-ethanol extraction procedures gave higher yields and purity than the acid-ethanol method. The extraction rates and protein contents obtained were 44.1 and 98.94%, respectively, for acetic acid extraction; 24.2 and 42.32%, respectively, for acidic-ethanol extraction; and, 56.8 and 94.88%, respectively, for alkaline-ethanol extraction. Wang and others (2009) surmised that acidic-ethanol was not strong enough to dissolve denatured proteins and that the extent of disulfide bond disruption is diminished at low pH. Furthermore, presoaking with a reducing agent in the glacial acetic acid procedure led to a higher extraction percentage. The Fourier transform infrared spectroscopic analysis of the samples revealed that kafirin extracted by acetic acid and alkaline-ethanol had a greater distribution of α-helices and random coils than kafirin extracted by acidic-ethanol.

Additionally, only kafirin extracted with acidic-ethanol had β-sheet conformations. Wang and others (2009) inferred that the presence of β-sheets was due to the higher extraction temperature used in the acidic-ethanol method. Differential scanning calorimetry showed a glass transition peak at around 230$^{o}$C for all powdered protein extracts. Size exclusion chromatography revealed that acetic acid and acidic-ethanol extraction methods extracted more of high molecular weight polymeric proteins (~20-30 kDa) than the alkaline-ethanol method. Additionally, γ-kafirins were observed only in the alkaline-ethanol extracts. Reversed phase high-performance liquid chromatograms of all the extracts from sorghum DDGS were not as sharp as those seen in kafirins extracted directly from sorghum endosperm, indicating the possibility of protein degradation or modification due to the extreme conditions of processing and extraction. A summary of all the protein extraction procedures discussed above is shown in Table 2.1.

## 2.6 CONCENTRATION OF SORGHUM PROTEINS

Kafirins, without any modification, exhibit limited functionality. At present, available literature focuses mainly on kafirin extraction and its application in films. Scientists find it challenging to develop economical, food compatible and non-toxic extraction procedures that can be scaled up to a commercial process because of the propensity of these proteins to form extensively aggregated networks and tightly bound structures. Without first developing a suitable kafirin concentrate, modifying its properties will be even more challenging. However, most cereal protein concentration procedures that have been described in literature are either based on cereals other than sorghum, or are secondary processes with the main goal being separation of starch for downstream applications such as ethanol production.

A protein concentrate can be developed by treating flours with α-amylase in order to breakdown starch, a process called liquefaction, and then washing out the degraded material. Liquefaction of flour yields concentrates containing protein, fiber and lipids. This process does not use harsh and toxic chemicals, making the end product safe for food use. Typically, liquefaction is used to produce maltodextrins and sugars for ethanol production from starch. However, some researchers have used this process to produce protein concentrates. Paredes-Lopez and others (1990) made protein concentrates from amaranth flour with 26-28% protein by treating flour with either heat-stable α-amylase or glucoamylase. Shih and Daigle (1997) treated rice flour with a heat-stable α-amylase and obtained a concentrate with 65% protein. When they

further treated the concentrate with cellulase and hemicellulase, protein content was raised to 76%. Paraman and others (2006) also used enzymatic treatment of rice with a heat stable α-amylase and a cellulase to isolate proteins up to 86% concentration. Barrows and others (2009) applied for a patent describing the production of protein concentrate from starch containing grain or oil seed using enzymes that hydrolyze starch, maltodextrins and β-glucans.

### 2.6.1 *Extrusion liquefaction*

Conventional liquefaction is a batch process wherein a 30-40% w/w solids starch slurry adjusted to pH 6.0 to 6.5 is jet-cooked together with a thermostable α-amylase at 103-105°C for 5 min., or at 95°C for 1-2 h (Bigelis 1993). When flours (e.g., rice, corn and sorghum flours) are used as starting materials, this step can be followed by treatment with other carbohydrate-hydrolyzing enzymes such as glucoamylase, cellulase and hemicellulase as discussed above. To speed up the process, liquefaction can be combined with extrusion. Extruding starches and flours degrades and gelatinizes starch thermo-mechanically, making the substrate more amenable to enzymatic attack. Meagher and Grafelman (1999) published a patent describing the liquefaction of cereal grain starch using an extruder. The inventors described how wet or dry milled corn was extruded in a single-screw extruder and then passed through a static mixer (attached to the extruder) where thermostable α-amylase was added. Liquefaction ensued in the post-extrusion reactor, a barrel attached to the end of the static mixer, and in a receiving tank where the extrudate was held at 90°C for 15 min. Vasanthan and others (2001) also used extrusion-enzyme liquefaction for starch dextrinization in barley flours. These authors extruded barley flours in a twin-screw extruder and found the optimum processing temperature that maximized α-amylase activity while minimizing its inactivation was 100°C. Materials extruded with 50% moisture (flour dry weight basis) had the highest dextrose equivalent due to increased starch gelatinization and enzyme hydrolysis. They also found that degree of hydrolysis at the same moisture and temperature at 4% α-amylase was twice that at 2% α-amylase.

As for other cereal flours and starches, liquefaction of sorghum flour is also typically carried out to produce sugars for ethanol production (Perez-Carrillo and others 2008; Wu and others 2007; Corredor and others 2006). Supercritical fluid extrusion, a modification of the conventional extrusion process, has also been used for cooking whole sorghum flour prior to liquefaction for ethanol production (Zhan and others 2006). These authors found that extrusion

with supercritical carbon dioxide effectively disrupted the protein matrix surrounding starch, making the material easier to liquefy. The above studies point towards the utility of the extrusion process for concentration of sorghum proteins, and a method for concentrating insoluble sorghum proteins was recently developed using decorticated sorghum flour by extrusion-enzyme liquefaction (de Mesa and others 2009; de Mesa-Stonestreet and others 2008) (Fig. 2.4). Decorticated sorghum flour was liquefied with a thermostable α-amylase in either, or both, the extruder or batch mixer. Then, the liquefied material was boiled to inactivate the enzyme, washed and centrifuged. The protein concentrate in form of the precipitate was then collected and freeze-dried. It was thought that the extrusion process disrupted the sorghum protein bodies and glutelin matrix and simultaneously brought about starch gelatinization, which in turn facilitated liquefaction by a thermostable α-amylase. This was a relatively speedier process that yielded concentrates with higher protein contents (up to 80%), and made sorghum proteins more digestible. Further research is needed to optimize the extrusion and enzyme liquefaction processes and characterize and modify the isolated proteins for use in foods. Elkhalifa and others (2009) also recently reported a preparation of sorghum protein-enriched flour that involved digesting boiled whole grain sorghum flour with α-amylase overnight at ambient temperature.

## 2.7   MODIFICATION OF SORGHUM PROTEINS

Sorghum use in food is limited by its poor digestibility and lack of functionality, which are exacerbated during wet-cooking. Protein modification studies have been undertaken in attempt to overcome these problems, and these can be classified into three broad categories – biochemical/chemical, enzymatic and thermo-mechanical.

### 2.7.1   *Biochemical/chemical modification:  Fermentation*

Fermentation of sorghum porridge improved protein digestibility (Elkhalifa and others 2006; Taylor and Taylor 2002; Yousif and El Tinay 2001; El Khalifa and El Tinay 1995). Yousif and El Tinay (2001) found a marked increase in sorghum *in vitro* protein digestibility (from 51.8 to 75.6%) after 24 h of fermentation. After 24 h, the albumin and globulin fractions decreased, the kafirin content increased, but there was no clear trend in the changes occurring in the crosslinked kafirin, glutelin-like, true glutelin and nonextractable protein fractions. Hence, while these authors purport that enhanced *in vitro* protein digestibility after fermentation was due

to the partial degradation of complex storage proteins into simpler and more soluble products, the changes in the protein fractions do not clearly indicate how this came about.

Taylor and Taylor (2002) also observed a decrease in water soluble proteins in sorghum flour accompanied by higher *in vitro* protein digestibility as a result of a fermentation. As such, these authors inferred that rather than being broken down into smaller subunits, prolamins and glutelins underwent structural changes during fermentation which made them more accessible to pepsin digestion. Furthermore, this structural change was attributed to the reduction in pH from about 6 to 3.4 and to the increase in titrable acidity due to lactic acid fermentation. Most of these changes took place during the first day and only slight variations were observed over the next 5 days of the study. While fermented sorghum flours had higher *in vitro* protein digestibility than raw sorghum flour, cooking the fermented flours still resulted in lower *in vitro* protein digestibility (11.5%) in comparison to raw sorghum flour. However, the fermented and cooked sorghum flour had greater protein digestibility than cooked sorghum flour that was not fermented (2% digestibility). While Taylor and Taylor (2002) asserted that changes occurred in both prolamins and glutelins, Elkhalifa and others (2006) reported that proteolysis of the glutelin fraction occurred and that the kafirin protein bodies remained intact (as observed with scanning electron microscopy). Furthermore, these authors also reported that insoluble protein aggregates still formed in fermented sorghum flour after cooking in boiling water.

Fermentation affected not only protein digestibility but also its functional properties (Elkhalifa and others 2005). For example, it was found that fermentation shifted the solubility of sorghum proteins by 2 pH units, with unfermented sorghum flour having a minimum solubility at pH 4 and fermented samples having a minimum solubility at pH 6 (Elkhalifa and others 2005), which suggests modifications of the proteins during fermentation. Fermentation increased the ability of sorghum flour to act as a gelling or firming agent, which is useful in foods like puddings. Although water-binding capacity of sorghum flour decreased, its oil-binding capacity increased by 7% as a result of fermentation. A reduced water-binding capacity makes it desirable for making thinner gruels, while a higher oil-binding capacity makes is useful in foods requiring oil retention. The emulsifying capacity of sorghum flour peaked to 52.83% (an increase of 7%) and emulsifying stability was 52.11% (9% increase) after 16 h of fermentation. This functional property makes it applicable in mayonnaise, salad dressings and frozen desserts. Both fermented and unfermented sorghum flour showed no foaming capacity.

The effects of fermentation on sorghum protein modification were also seen in bread. Schober and others (2007) studied the changes taking place in sorghum proteins in gluten-free sorghum bread undergoing sourdough fermentation. The authors reported that sourdough fermentation brought about a more stable crumb structure in bread based on the observation that gluten-free sorghum bread that underwent sourdough fermentation had no hole in the crumb and had a higher loaf height than breads without sourdough fermentation. Additionally, its hardness values in texture profile analysis (TPA) showed that it had a softer crumb and that it staled at a slightly slower rate. Their experiments ruled out that chemical acidification brought about these beneficial changes. Instead, evidence pointed toward proteolysis as the primary mechanism. Confocal scanning laser microscopy revealed degradation of protein aggregates in sourdough-fermented bread. In contrast, bread with the same formula but without sourdough fermentation had some protein aggregation, and bread that was chemically acidified to the same pH as sourdough-fermented bread (5.2) had even more heavily aggregated proteins. Schober and others (2007) inferred from the SEC data that many proteins were degraded into fragments small enough that crosslinking upon baking was no longer possible.

### 2.7.2 Biochemical/chemical modification: Protein-polysaccharide conjugation

Babiker and Kato (1998) conjugated sorghum protein with dextran or galactomannan to improve its functional properties. The authors first extracted sorghum proteins in an aqueous alkaline (pH 8) medium containing 2-ME. Conjugation was carried out by first preparing a 10% mixture of sorghum protein with either dextran or galactomannan at a ratio of 1:5, which was then freeze-dried. The powdered mixtures were heated to $60^{o}C$ at 70% relative humidity in a dessicator containing saturated potassium bromide solution for 7 days. Both sorghum protein-dextran and sorghum protein-galactomannan conjugates were 90-95% soluble at all pH levels, even when heated to $90^{o}C$. Emulsifying capacity of the conjugates was almost twice that of sorghum protein alone, and the dextran conjugate was superior to the galactomannan conjugate. Stabilities of the emulsions with the dextran and galactomannan conjugates were 10 and 7 times better, respectively, than sorghum protein alone. It is important to note, though, that because an aqueous alkaline medium was used to extract the proteins, albumins, globulins, and possibly some glutelins, rather than kafirins, may be the proteins primarily extracted. Since albumins and globulins are initially soluble in water or saline solutions, this modification may not be effective

for kafirins, and due to the low levels of albumins and globulins in sorghum, this procedure would likely yield only relatively low levels of modified proteins from a given batch of sorghum flour.

### 2.7.3  Biochemical/Chemical modification:  Reducing agents

Reducing agents have been used to modify both the *in vitro* digestibility (Choi and others 2008; Elkhalifa and others 1999; Zhang and Hamaker 1998; Arbab and El Tinay 1997; Rom and others 1992; Hamaker and others 1987) and extractability (Bean and others 2006; Park and Bean 2003) of sorghum proteins.  Of the reducing agents tested, sodium metabisulphite, glutathione and L-cysteine are suitable for some food use.   These reagents work by breaking  disulfide linkages in kafirins and the protein matrix.  Using scanning electron microscopy, Rom and others (1992) showed that boiling a sorghum flour suspension with sodium bisulfite for 20 min resulted in a breakdown of the protein matrix and pitting of the protein bodies.  Using confocal laser scanning microscopy, Choi and others (2008) depicted breakdown of the protein matrix and increased starch digestion resulting from cooking a sorghum flour suspension with sodium bisulfite.  Ezeogu and others (2008) also used confocal laser scanning microscopy to show the effect of cooking with and without 2-ME on the formation of protein matrices in vitreous and floury sorghum endosperm flour.  These authors found that cooking without a reducing agent resulted in discontinuities and an expansion of the protein matrix in floury sorghum endosperm flour but not in the vitreous fraction, indicating a greater degree of protein crosslinking in the latter.  On the other hand, cooking with 2-ME reduced the density of the web-like protein network in both floury and vitreous endosperm flours due to the breakage of disulfide bonds.

### 2.7.4  Biochemical/chemical modification:  Enzymatic hydrolysis

Enzymes have advantages over chemical methods of protein modification.  First, reaction rates are fast and highly specific and conditions are generally mild, which limits damage to the nutritional quality of proteins and reduces production of toxic substances.  Second, and probably most important, enzymatic methods are generally safer than corresponding chemical based methods.   On the flip side, enzymes are not as cost effective as chemical processes.  Additionally, while specificity of enzymatic reactions is a commonly cited desirable attribute, it can also be a deterrent to its adoption because several enzymes may be needed to accomplish the job of a single chemical process.

Proteases are utilized to modify protein nutritional and sensory qualities (e.g., digestibility, allergenicity, bitterness, etc.) and protein functionality (e.g., solubility, dispersibility, foaming, water or oil binding, dough elasticity, etc.) (Kunst 2003; Nielsen 1997; Adler-Nissen 1986). In sorghum, proteolysis has been primarily used to isolate sorghum starch, to improve starch digestion for animal feeds and to improve starch hydrolysis for ethanol production, but there is limited research on enzymatic hydrolysis of kafirins. Yang and Seib (1995) used a type II protease from *Aspergillus oryzae* to aid in removing sorghum proteins during sorghum starch isolation. The authors were able to reduce the protein content from 0.7-1.1% (dry weight basis, db) to 0.5-0.6% db by treating sorghum starch with the protease. When isolating sorghum starch, Xu (2008) treated sorghum flour with pepsin, and achieved an almost complete hydrolysis of the proteins after 4 h. The isolated starch had 0.5% protein content. Xu (2008) also found that pepsin pre-treatment improved sorghum starch digestibility. Similarly, Mezo-Villanueva and Serna-Saldivar (2004) were able to achieve greater starch recovery after steeping sorghum flour for 24 and 48 h using Neutrase™, a neutral metalloprotease requiring $Zn^{2+}$ and $Ca^{2+}$ for its activity. Zhang and Hamaker (1998) found an increase in starch digestibility of cooked sorghum flours by 7-14% when sorghum flours were pre-treated with pepsin. Benmoussa and others (2006) also found that sorghum starch digestion profile over a 12 h period was significantly improved by pepsin pre-treatment of raw sorghum flour for feed use.

Sorghum flour has also been treated with protease to facilitate starch liquefaction. Perez-Carrillo and Serna-Saldivar (2007) treated decorticated and whole sorghum flour slurry with Neutrase™. The slurry containing Neutrase™ was heated to $60 \pm 1^{o}C$ for 30 min prior to the addition of a thermostable α-amylase. Their experiments showed that protease pre-treatment resulted in a 44.7% reduction in liquefaction time. Using the same protease pre-treatment procedure described above, Perez-Carillo and others (2008) found that decorticated sorghum flour treated with protease had ~50% more reducing sugars than its untreated counterpart and that fermentation time was reduced from 60 to 22 h. However, none of the above studies on sorghum starch isolation and liquefaction investigated the impact of proteases on structure, digestibility or functionality of the residual proteins.

Ng'andwe and others (2008) treated raw and wet-cooked sorghum flour with a combination of an aminopeptidase (Flavourzyme™) and potassium metabisulfite at $40^{o}C$ for 7 h. An aminopeptidase rather than a sulphydryl protease was specifically used because the latter

enzyme is inactivated by potassium metabisulfite. Confocal laser scanning micrographs and transmission electron micrographs revealed that the exogenous protease alone digested the glutelin protein matrix surrounding the starch granules in both raw and cooked sorghum flours, and that the presence of both enzyme and potassium metabisulfite in the mixture brought about the reduction of not only the glutelin matrix, but also the exterior parts of the protein bodies. SDS-polyacrylamide gel electrophoretic patterns also showed that potassium metabisulfite was effective in reducing kafirin polymers and oligomers into monomers.

Kamath and others (2007) hydrolyzed isolated α-kafirin in Tris-HCl buffer (pH 7.6) containing SDS with chymotrypsin. Hydrolysis was carried out at $37^{o}$C for 4 h with 40 μg chymotrypsin/ mg α-kafirin. The authors obtained a hydrolysate rich in peptides that inhibited (*in vitro*) angiotensin I converting enzyme (ACE). Inhibiting ACE is useful in treating high blood pressure (reviewed by Kamath and others 2007).

## 2.7.5 *Biochemical/Chemical modification: Deamidation*

Deamidation is known to improve solubility, emulsification activity and stability and foaming of plant storage proteins (Haard 2001). This reaction is characterized by the conversion of the amide groups of asparagine and glutamine to carboxyl groups, which can be accomplished chemically (under acidic or basic conditions) or enzymatically. Acidic deamidation with 0.05N HCl for 15-30 min at $95^{o}$C was successful in improving the solubility of zein (Casella and Whitaker 1990). On the other hand, alkali deamidation at pH 11.0 and $25^{o}$C was conducted on rice protein isolates by Paraman and others (2007). A procedure for the enzymatic deamidation of food proteins, primarily soy, corn, rice, egg and milk proteins, was described in the patent by Hamada and Marshall (1992). This patent described the optimization of deamidation by first heat denaturing or enzymatically hydrolyzing proteins prior to deamidation with peptidoglutaminase. The denaturation and/or hydrolysis steps were necessary in opening the protein structures to provide more sites for peptidoglutaminase to act on. While deamidation has not been used in sorghum, its success in zein and corn gluten indicates that it can also be used in modifying kafirins. However, pre-treatment of kafirins by thermo-mechanical and/or chemical means may be necessary because of the nature of sorghum protein bodies.

### 2.7.6 Thermo-mechanical modification: Irradiation

Fombang and others (2005) used γ-irradiation to modify sorghum proteins. Sorghum porridge cooked with sorghum flour irradiated at 10 kGy showed a significantly higher *in vitro* protein digestibility than that cooked with untreated sorghum flour. The authors hypothesized that irradiation cleaved kafirin disulfide bonds and fragmented proteins, leading to reduced disulfide crosslinking during cooking. This more open structure would have been more susceptible to proteolytic digestion. Digestibility of porridge made from flour irradiated at 50 kGy, however, was lower than that of porridge made from 10 kGy irradiated flour but higher than that of porridge made with untreated flour. Fombang and others (2005) attributed the reduced digestibility to crosslinking and aggregation at higher doses and to production of Maillard products that inhibit proteolytic activity.

### 2.7.7 Thermo-mechanical modification: Extrusion

Extrusion of sorghum flour improves its protein digestibility. MacLean and others (1983) found that decortication and low moisture extrusion improved digestibility of sorghum when prepared into porridge and fed to preschool children. Mertz and others (1984) established that after boiling with water, dried and ground sample of decorticated sorghum flour extruded at low moisture and 350$^\circ$C had 22% higher *in vitro* protein digestibility than decorticated sorghum flour. In a study by Fapojuwo and others (1987), extrusion also raised *in vitro* protein digestibility of sorghum by about 30%. These authors also saw that there were no significant differences in digestibilities between 2 moisture levels (15 and 25%), but that increasing screw speed from 50 to 125 rpm, as well as raising temperature (50, 125 and 200$^\circ$C), significantly increased protein digestibility. Fapojuwo and others (1987) also reported that pre-treatment of sorghum grain with 4% calcium hydroxide (pH 11.0) further increased the protein digestibility of extruded sorghum grain. Dahlin and Lorenz (1993) processed whole sorghum flour in a single-screw extruder and evaluated the effect of feed moisture (15, 25%), processing temperature (100$^\circ$ and 150$^\circ$C) and screw speed (100 and 150 rpm) on *in vitro* protein digestibility. Their results revealed that extruding with 15% feed moisture at 150$^\circ$C and 100 rpm were the optimum conditions for yielding extrudates with high *in vitro* protein digestibility. While they found that extruding at low moisture and high temperature raised protein digestibility, they saw that the effect of raising screw speed was less obvious. Dahlin and Lorenz (1993) explained that

extruding at 15% moisture gave better digestibility values probably by reducing the reaction rate of degradative processes. These degradative processes, however, were not specified. Additionally, these authors said that extruding at 150°C instead of 100°C favored digestibility due to greater denaturation of protein and inactivation of enzyme inhibitors.

Hamaker and others (1994) studied the *in vitro* protein digestibility and protein distribution of cooked flour porridges of decorticated only and decorticated and extruded sorghum flours. Their study showed that extrusion of decorticated sorghum flours raised protein digestibility by 18%. They also found that the percentage of prolamins extractable by 60% t-butanol increased by 12% and that the percentage of prolamins extractable by t-butanol with a reducing agent decreased by 17%. This indicated a shift in protein distribution towards the more digestible fraction, which could explain the improvement in protein digestibility. However, this was also accompanied by a shift in the glutelin fraction to the nonextractable fraction. The latter, though, did not appear to affect protein digestibility.

Batterman-Azcona and others (1999) studied the relationship between extrusion and maize protein body disaggregation. They found that α-zeins remained intact under mild processing conditions and were released only at a specific mechanical energy (SME) of about 100 kJ/kg. At 165 kJ/kg, the protein bodies were completely disrupted, α-zein was dispersed and it was inferred that these formed protein fibrils. Even though kafirins bear a high degree of homology to zein, the extensive crosslinking that occurs in kafirins during cooking may require a higher specific mechanical energy to open the protein bodies (Ezeogu and others 2008; Hamaker and others 1986).

## 2.8 CONCLUSIONS

Sorghum is an attractive raw material and a good source of protein for wheat-free products due to the neutral flavor and color of specific varieties, low allerginicity and its ability to grow in drought-like conditions. Although sorghum has been mainly used for animal feed in the U.S., it has a huge potential for food use, including as a source of concentrated proteins for incorporation in gluten-free foods. However, the acceptability of sorghum and its proteins as food ingredients depends not only on their nutritional characteristics, but also on sensory and technological properties. At present, a huge gap exists between the present and desired

nutritional and functional characteristics of sorghum and its proteins, limiting their use in foods. Sorghum proteins have low digestibility, which is further reduced during cooking with high moisture. Furthermore, unlike wheat proteins, sorghum proteins are not highly functional. Concentration and/or modification of sorghum proteins could be one way to address this challenge. However, research to date has focused on sorghum protein extraction with non-food compatible and unsafe chemicals, and incorporation of kafirins in highly demanded staples like bread and pasta is scant.

Thus, areas for future work include development of economical, food-compatible and safe methods for concentrating and/or extracting sorghum proteins, especially kafirins, that can be scaled up to a commercial level and modification of the functional properties of kafirins in order to increase the scope of their applications in foods.

## 2.9 ACKNOWLEDGEMENT

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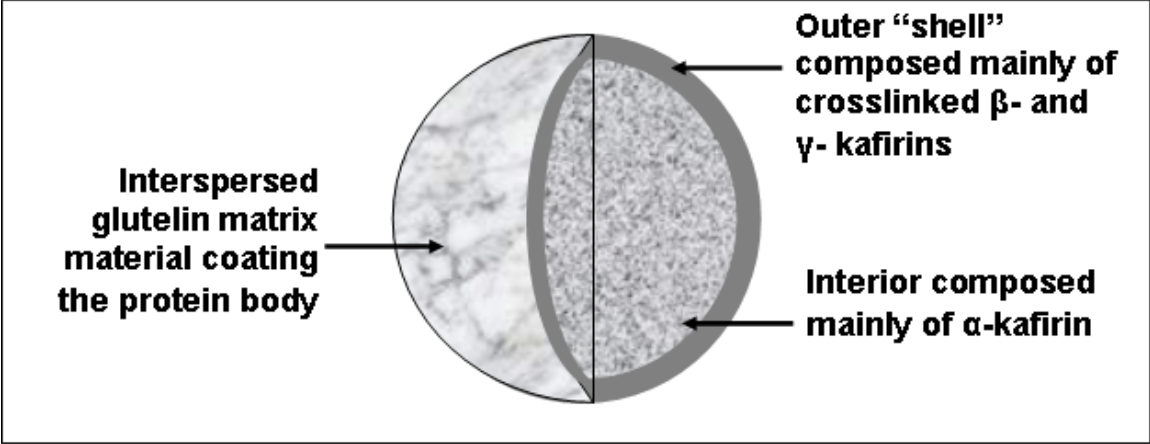
**Fig. 2.1 Schematic of a sorghum protein body**

**Protein bodies**

**Starch granule**

**Glutelin protein matrix**
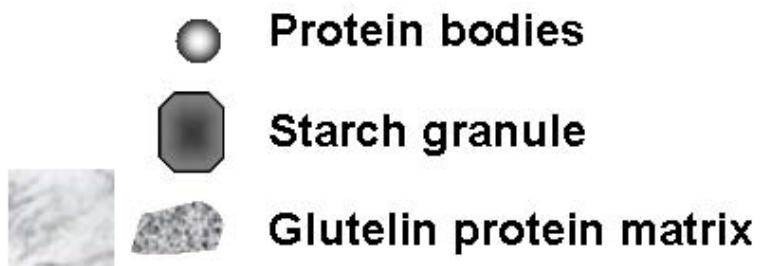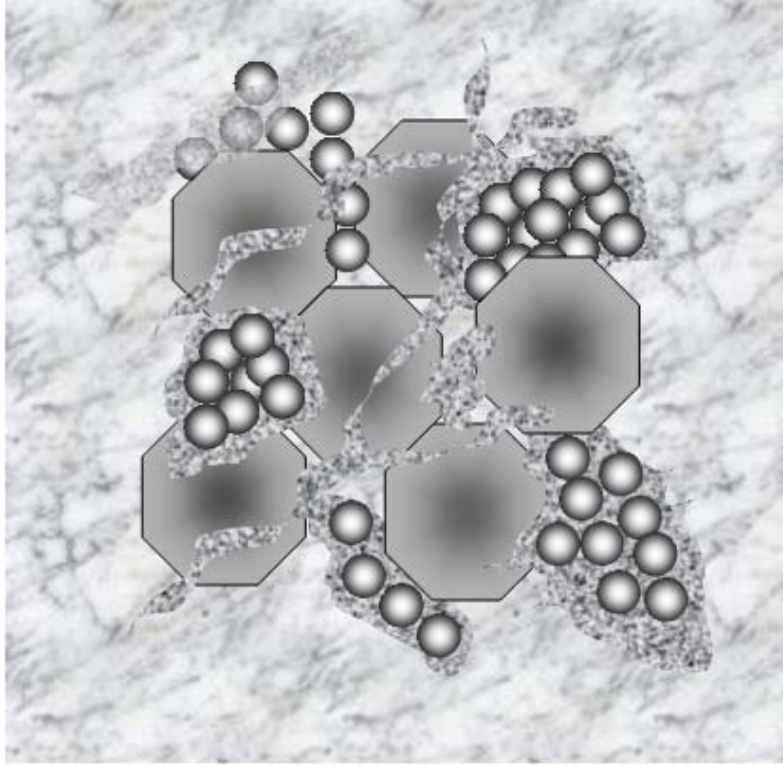
Protein bodies and starch granules are
embedded in the glutelin matrix.

**Fig. 2.2 Sorghum protein bodies in relation to starch and the glutelin matrix**

Sorghum grain

STEEPING

24-48 h; 48-55$^O$C; grain sorghum:water = 1:1.5 to 1:5;
Some additives: 0.05-0.30%(w/w) SO$_2$; 0.4-1.4% (w/w) lactic acid; protease; cell-wall-degrading enzymes

Steep liquor          Steeped kernels

Water ⟶ COARSE GRINDING

Slurry

Water ⟶ FILTERING

Germ & bran/coarse fiber ⟵          Throughs

Supernatant ⟵ SEPARATION (centrifugation or settling)

Sediment

Water ⟶ FINE GRINDING

Slurry

Water ⟶ FILTERING

Fine fiber ⟵          Throughs

Supernatant ⟵ SEPARATION (centrifugation or settling)

Sediment

SEPARATION

Dry Protein Fraction ⟵ DRYING (20-60$^O$C) ⟵ Protein          Starch ⟶ WATER WASHING ⟶ DRYING (40-50$^O$C) ⟶ Dry Starch Fraction

**Fig. 2.3 Sorghum wet-milling process**
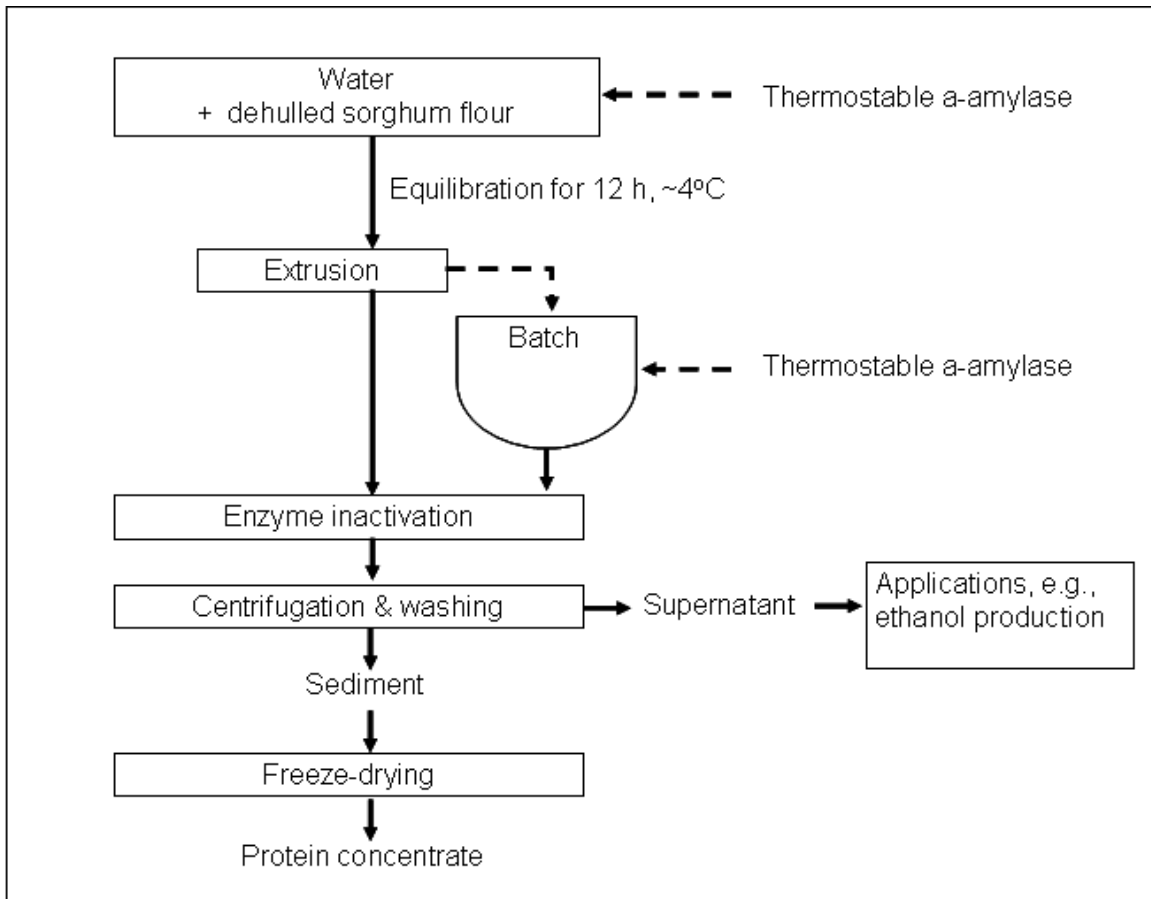
**Fig. 2.4 Extrusion-liquefaction process for producing sorghum protein concentrate**

**Table 2.1 Methods of Protein Extraction and Classification**

| Method | Reagent | Function/ Interactions broken | Proteins obtained |
|---|---|---|---|
| Osborne Procedure (Wrigley and Bekes 2001; Hamaker and others 1995; Taylor and others 1984b; Virupaksha and Sastry 1968;Osborne 1907) | distilled water, 1% NaCl solution | breaks non-covalent electrostatic interactions | albumins globulins |
| | aqueous alcohol (e.g., 70% v/v ethanol, 60% t-butanol, 70% isopropanol) | weakens hydrophobic interactions and acts as a solvent | prolamins |
| | Acidic or basic solution (e.g., 0.4% NaOH, alkali borate buffer, pH 10.0) | non-covalent electrostatic interactions | glutelins |
| Landry-Moureaux Sequential Extraction Procedure (Hamaker and others 1995) | 0.5 M NaCl solution | breaks hydrophilic interactions | albumins and globulins |
| | 60% t-butanol | weakens hydrophobic interactions and acts as a solvent | prolamins (kafirin-1) |
| | 60% t-butanol + 0.5% 2-mercaptoethanol (2-ME) | 2-ME is a reducing agent that breaks covalent disulfide bonds | prolamins (kafirin-2/ crosslinked kafirins) |
| | 12.5 mM alkali-borate buffer + 0.5% 2-ME | breaks non-covalent electrostatic interactions and disulfide bonds | glutelin-like proteins |

| Method | Reagent | Function/ Interactions broken | Proteins obtained |
|---|---|---|---|
| | 12.5 mM alkali-borate buffer + 2% sodium dodecyl sulfate (SDS) | SDS is an anionic detergent that breaks hydrogen bonds and hydrophobic interactions | true glutelins (supernatant); nonextractable proteins (precipitate; determined by nitrogen combustion) |
| Alkaline extraction (Wu 1978) | NaOH solution, pH 11.9 | break non-covalent hydrophilic interactions and acts as a solvent | possibly glutelin |
| | HCl solution, pH 4.8 | precipitate proteins | |
| Non-kafirin/ kafirin sequential extraction (Park and Bean 2003; Hamaker and others 1995) | 12.5 mM sodium borate buffer, pH 10.0 + 1% or 2% SDS + 2% 2-ME[1] | breaks non-covalent electrostatic interactions, hydrogen bonds, and disulfide bonds | total proteins[2] |
| | 60% t-butanol | non-kafirin precipitation | kafirins and non-protein nitrogen (supernatant); detergent-extractable non-kafirins (precipitate) |

| Method | Reagent | Function/ Interactions broken | Proteins obtained |
|---|---|---|---|
| | acetone (8:1 ratio, acetone to sample) | kafirin precipitation | kafirins (precipitate) |
| Sonication with ethanol (Bean and others 2006) | 70% ethanol (+ sonication)[3] | ethanol breaks hydrophobic interactions and solubilizes prolamins; sonication breaks crosslinks that hold large protein aggregates | kafirin monomers, crosslinked kafirins, lipids |
| | dilution of ethanol to 60% with water | precipitate lipids | kafirin monomers and crosslinked kafirins (supernatant) |
| | further dilution of ethanol (ranging from 50 to 30%)[4] | precipitates prolamins | kafirin monomers and crosslinked kafirins |
| Sonication with sodium borate and SDS (Zhao and others 2008; Park and others 2006) | 12.5 mM sodium borate buffer, pH 10.0 + 2% SDS (+ sonication; 10W, 30 s) | buffer breaks non-covalent electrostatic interactions; SDS breaks hydrogen bonds and hydrophobic interactions; sonication breaks large protein aggregates | detergent-extractable proteins, crosslinked proteins |

| Method | Reagent | Function/ Interactions broken | Proteins obtained |
|---|---|---|---|
| | 12.5 mM sodium borate buffer, pH 10.0 + 2% SDS + 2% β-ME | buffer and SDS function as above; β-ME breaks disulfide bonds | detergent-extractable proteins, crosslinked proteins |
| Polymeric protein sequential extraction using differential solubility and sonication (Ioerger and others 2007) | 12.5 mM sodium borate buffer, pH 10.0 + 2% SDS | breaks non-covalent electrostatic interactions, hydrogen bonds and hydrophobic interactions | soluble proteins (supernatant)[5] |
| | 12.5 mM sodium borate buffer, pH 10.0 + 2% SDS + sonication (10W, 30 s) | sonication breaks large protein aggregates; buffer functions as above | insoluble proteins (supernatant)[5]; heavily crosslinked residue protein (precipitate)[5] |
| Glacial acetic acid extraction of kafirins (Taylor and others 2005) | pre-soak for 16 h in 0.5% Sodium metabisulfite (SMS) at 25°C | breaks disulfide bonds | |
| | glacial acetic acid | breaks hydrophobic interactions | |
| | NaOH for adjusting pH to 5 | kafirin precipitation | kafirins |

| Method | Reagent | Function/ Interactions broken | Proteins obtained |
|---|---|---|---|
| Acidic-ethanol method (Wang and others 2009) | 70% ethanol, pH 2.0 + SMS | weakens hydrophobic interactions, breaks disulfide bonds and acts as a solvent | kafirins |
| Alkaline-ethanol method (Wang and others 2009; Emmambux and Taylor 2003) | 70% ethanol + 0.35% NaOH + 0.5% SMS | weakens hydrophobic interactions, breaks electrostatic and disulfide bonds, and acts as a solvent | kafirins |
| | dilution of ethanol to 40%, $-20^{o}$C | kafirin precipitation | |

Notes:

[1] Alternative reducing agents include dithiothreitol (DTT), tris(2-carboxyethyl) phosphine hydrochloride, glutathione and sodium metabisulfite (SMS)

[2]Alternatively, albumins and globulins can be removed with 1% NaCl solution prior to extraction of the remaining proteins (kafirins and glutelins).  This also allows isolation of the glutelin fraction after kafirins have been extracted.

[3]In some experiments, a reducing agent (SMS or glutathione or cysteine) was added.  SMS and glutathione extracted the most protein. The addition of reducing agents to ethanol was more effective in extracting proteins than sonication.

[4]NaCl may be added and pH may be lowered to 2.5.

[5]Soluble, insoluble and residue proteins are analogous to the kafirin-1, kafirin-2 and glutelin fractions of the Landry-Moureaux procedure.

# Chapter 3 - Extrusion-enzyme liquefaction as a method for producing sorghum protein concentrates[1]

## 3.1   ABSTRACT

A novel method was developed for concentrating proteins from sorghum flour utilizing a combination of extrusion and α-amylase treatment for starch liquefaction. A central composite design was used to optimize in-barrel moisture content (MC), enzyme concentration during extrusion (E1) and post-extrusion enzyme concentration (E2) in order to produce sorghum protein concentrates with high protein content (PC) and *in vitro* protein digestibility (D). Extrusion-enzyme liquefaction yielded concentrates with higher protein yield (82% db) and digestibility (66%) than batch liquefaction alone because extrusion promoted starch degradation and protein disaggregation. The optimum conditions for developing a sorghum protein concentrate with both high yield and digestibility were 32% MC, no E1, and 2.5% E2. The sorghum protein concentrate developed in this study can augment the nutritional value of gluten-free foods for individuals suffering from celiac disease and other forms of gluten and wheat intolerance.

## 3.2   NOMENCLATURE

CLSM – confocal laser scanning microscopy

D – *in vitro* protein digestibility (%)

% db - percentage dry basis

E1 – enzyme concentration during extrusion (% fwb)

E2 – enzyme concentration post-extrusion (% fwb)

EP – extracted protein

% fwb – percentage flour weight basis

IP – insoluble protein

MC – in-barrel moisture content (%)

PC – protein content (% db)

RP – residue protein

SME – specific mechanical energy (kJ/kg)

SP – soluble protein

## 3.3  INTRODUCTION

Celiac disease is an autoimmune reaction to the ingestion of the prolamin proteins found in wheat (gluten), rye (secalin) and barley (hordein).  It is a serious enteropathic condition that affects 1 in 133 Americans (Fasano and others 2003).  In addition, there are also individuals with varying levels of gluten sensitivity.  A life-long avoidance of gluten and similar proteins is the only recourse of these individuals.  A need exists to develop gluten-free foods, especially staples such as bread and pasta that are nutritious and palatable.  Unfortunately, the existing gluten-free food products are primarily starch-based and lack essential nutrients like protein, fiber, vitamins and minerals (Engleson and Atwell 2008).

Grain sorghum (*Sorghum bicolor* L. Moench) is safe for celiac patients (Ciacci and others 2007) and can be used as an alternative to wheat due to the use of white grained hybrids that do not impart an unusual color to the flour and have a neutral flavor.  Even then, the utilization of sorghum in gluten-free foods has been limited partly due to the morphological and chemical characteristics of its proteins which negatively impact their digestibility and functionality.  Sorghum grain has an average protein content of 11% (Lasztity 1996) and its proteins are classified into prolamin (kafirin) and non-prolamin proteins (albumin, globulin, glutelin).  Kafirin proteins are the major storage proteins that account for 77 to 82% of the endosperm proteins and are categorized as α, β, γ, δ based on molecular weight and solubility (Belton and others 2006).  Th e α-kafirins minimally participate in intermolecular crosslinking; β- and γ-kafirins form extensive crosslinks; and, δ-kafirins remain to be fully characterized.  Kafirins form rigid protein bodies that are tightly embedded in the protein glutelin matrix (Taylor and others 1984).  These protein bodies have a rigid outer shell composed of crosslinked β- and γ-kafirins that encapsulate monomeric α-kafirins (de Mesa-Stonestreet and others 2010).  The encapsulation of α-kafirins in rigid protein bodies enveloped by the protein glutelin matrix make it difficult to disrupt and digest (Wong and others 2009; Oria and others 1995; Hamaker and others 1994).  Raw, decorticated sorghum flour has *in vitro* protein digestibility of 65 to 80% (Duodu and others 2002; Weaver and others 1998; Oria and others 1995).   Wet cooking enhances protein crosslinking, making kafirins more resistant to digestion, thereby reducing their nutritional value (Duodu and others 2002, 2003; Hamaker and others 1986).  *In vitro* protein digestibility of cooked, decorticated sorghum flour is 40-44% (Duodu and others 2002; Oria and

others 1995).   Kafirins also have poor rheological properties due to extensive disulfide crosslinking of kafirin monomers (Oom and others 2008).

Several small-scale laboratory procedures have been developed to isolate kafirins.  These procedures were recently reviewed in detail by de Mesa-Stonestreet and others (2010).  Kafirins have been isolated with aqueous alcohol (Bean and others 2006; Park and Bean 2003; Hamaker and others 1995; Osborne 1907), glacial acetic acid (Taylor and others 2005), and alkaline sodium borate buffer with detergent (sodium dodecyl sulfate) ( Zhao and others 2008a; Ioerger and others 2007; Park and others 2006).   Reducing agents (e.g., sodium metabisulfite, β-mercaptoethanol and glutathione) (Taylor and others 2005; Park and Bean 2003) and sonication (Zhao and others 2008b; Ioerger and others 2007; Bean and others 2006; Park and others 2006) have also been used to increase protein extraction rates.   While the abovementioned methods produced kafirins useful for characterization, these were only bench-top experiments with low yields and little scale-up potential.   In addition, most of these procedures used non-food-compatible reagents.

Extrusion-enzyme liquefaction is a novel method for concentration of proteins from sorghum flour and has commercial promise as a large-scale food-compatible process (de Mesa-Stonestreet and others 2010).  In comparison to traditional batch liquefaction, extrusion is a high throughput process, and its mechanical action simultaneously degrades starch and disrupts sorghum proteins that limit starch gelatinization.  The extrusion step makes starch liquefaction and removal easier and faster, potentially resulting in a highly pure sorghum protein concentrate that has good digestibility and improved functional properties with regard to food and beverage applications.  Batterman-Azcona and Hamaker (1998) have shown that extrusion disrupts maize protein bodies, which are homologous to sorghum proteins.  Moreover, as reported in other sorghum based applications (Hamaker and others 1994; Dahlin and Lorenz 1993; Fapojuwo and others 1987; Mertz and others 1984; MacLean and others 1983), mechanical shear during extrusion improves protein digestibility.    While extrusion-enzyme liquefaction has been used to produce sugars from cereal starches and tubers for ethanol production (Solihin and others 2007; Zhan and others 2006; Vasanthan and others 2001; Meagher and Grafelman, 1999; Curic and others 1998; Govindasamy and others 1997a; Chouvel and others 1983), this process has not been utilized in the past for protein concentration.  The specific objectives of this study were to optimize extrusion in-barrel moisture content and the α-amylase level during and post-extrusion

for obtaining sorghum protein concentrates with high protein percentage and *in vitro* digestibility.


## 3.4 MATERIALS AND METHODS

### *3.4.1 Materials*

A white sorghum hybrid Fontanelle 1000 was used for this experiment. Decortication and milling of sorghum grain was done in a commercial-scale facility (AgVanced Enterprises, New Cambria, KS). Sorghum grain was decorticated to remove 12.5% of kernel weight using a vertical pearling machine. The mean particle size of sorghum flour was 119 μm as determined using a laser diffraction particle size analyzer (LS™ 13 320, Beckman-Coulter, Inc., Miami, FL). The flour had 9.68% db protein; 1.16% db fat; 0.69% db ash; and 88.47% db carbohydrates (including 0.22% db crude fiber).

A liquid preparation of thermostable α-amylase from *Bacillus licheniformis* with a declared activity of 240 KNU-S/g (Liquozyme SC DS, Novozymes, Franklinton, NC) was used to bring about starch liquefaction. The optimum conditions for enzyme activity were pH 5.7-6.0 and 82-86°C.

### *3.4.2 Experimental Design*

A central composite design (CCD) with three factors and three levels each was used. The factors studied were in-barrel extrusion moisture content (MC), enzyme concentration in the extruder (E1) and enzyme concentration post-extrusion (E2). The experimental design was generated using the Active Design of Experiments (SAS ADX) feature of SAS 9.1.3 (SAS Institute, Cary, NC). The design points generated by SAS ADX are given in Table 3.1. For MC, the levels varied from 14% to 50% flour wet basis (fwb), with 32% fwb as the central point. Factors E1 and E2 ranged from 0 to 10% fwb, with 5% fwb as the central point. The response variables were % protein content (PC) and % *in vitro* protein digestibility (D).

A completely randomized design with ten supplementary extrusion-enzyme liquefaction treatments were conducted at three MC levels (17, 32 and 50% fwb) with and without enzymes. Additional batch liquefaction experiments, with enzyme addition levels from 0 to 15% fwb, were conducted to serve as control. These treatments are summarized in Table 3.2 and Table 3.3 were

designed to supplement the information gained from the central composite design and help explain the underlying factors affecting the extrusion-enzyme liquefaction process. It was not possible to extrude sorghum flour at MC less than 17% fwb without enzymes given the extrusion conditions used, so 17% fwb was the lowest in-barrel moisture selected for the supplementary treatments. The supplementary extrusion- and batch liquefaction treatments were duplicated.

Protein solubility test was performed for treatments selected from the CCD, supplementary and batch experiments. The extruded treatments chosen represented low, middle and high PC and D values. Batch liquefaction with 10% fwb enzyme was chosen because it had the PC and D values that most closely matched those of the extruded treatment with the highest PC. Untreated sorghum flour was included for reference.

Treatments in the text and figures are described as MC-E1-E2. For example, 14-5-5 for treatment extruded with 14% fwb MC and 5% fwb E1, and liquefied post-extrusion with 5% fwb E2.

### 3.4.3  Extrusion-Enzyme Liquefaction

The moisture content of sorghum flour was adjusted to the desired level (Table 3.1 and Table 3.2) by mixing sorghum flour with water in a KSM5 Kitchen Aid mixer (St. Joseph, MI), taking the initial moisture of the flour into account. The hydrated flour was placed in sealed plastic bags and stored overnight at $4^{o}C$ for equilibration prior to extrusion. In treatments where α-amylase was added in the extruder, the enzyme was added to the water used for adjusting flour moisture content. The amount of liquid enzyme added was taken into consideration when adjusting flour moisture content. The hydrated flour mixtures were extruded using a laboratory scale twin-screw extruder (M-18, American Leistritz, Somerville, NJ) with screw diameter of 18 mm and screw length to diameter ratio of 29:1. At the discharge end, a 19 mm long spacer with a temperature probe attachment and a 2 mm diameter circular die were used. The screw configuration and barrel temperature profiles are shown in Fig. 3.1. Feed rate, controlled with a volumetric feeder screw, varied between 1.48 to 2.25 kg/h. The extruder screw speed was kept constant at 350 rpm. For treatments without post-extrusion liquefaction, α-amylase was inactivated by adjusting the water-to-dry matter ratio to 80:20, reducing pH to 3.0 and then boiling for 20 min. For treatments requiring post-extrusion liquefaction, water was added to the extrudates to achieve an 80:20 water-to-dry matter ratio before adding the required amount of α-

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amylase. The mixture was then held for 1 h at 82$^{o}$C with constant agitation in a water bath and, subsequently, the enzyme was inactivated as previously described. Batch liquefaction of the control treatments was carried out in the same manner as the extrudates that underwent post-extrusion liquefaction.

Specific mechanical energy (SME) was computed for the extrusion process as follows (de Mesa and others 2009; Onwulata and others 1994):

$$SME(kJ/kg) = \frac{\left(\dfrac{\tau - \tau_0}{100}\right) \times \dfrac{N}{N_{rated}} \times P_{rated}}{\dot{m}} \qquad (1)$$

where, $\tau$ is the % rated motor torque (13 to 66%); $\tau_0$ is the no load motor torque (13%); $N$ is the screw speed (350 rpm); $N_{rated}$ is the rated screw speed (500 rpm); $P_{rated}$ is the rated motor power (2.2 kJ/s); and $\dot{m}$ is the mass flow rate (0.0004 to 0.0006 kg/s).

### 3.4.4 Protein Concentration

After enzyme inactivation, the mixture was centrifuged (4500$g$, 30 min); the sediment was collected and washed thrice with distilled water; centrifuged as above; and, lyophilized. Fig. 3.2 illustrates the extrusion-enzyme liquefaction and protein concentration processes.

Prior to subsequent analyses, all samples (protein concentrates, and raw and extruded flours) were ground and sifted through a Tyler No. 60 sieve to achieve particle size <250 μm. Overs were re-ground until all particles passed through the sieve. This procedure reduced variation between treatments and improved reproducibility of results, especially when performing the protein digestibility assay.

### 3.4.5 Proximate Analyses

The proximate composition of sorghum flour and sorghum protein concentrates was determined using standard methods (AOAC 2010; AOCS 2009). This included determination of moisture (135$^{o}$C for 2h; AOAC 930.15), crude protein (based on nitrogen by combustion, 6.25X; AOAC 990.03), crude fat (petroleum ether extract method; AOAC 920.39), ash (600$^{o}$C for 2h; AOAC 942.05), crude fiber (filter bag technique utilizing $H_2SO_4$ and NaOH digestion for Ankom 200 Fiber Analyzer, Ankom Technology, Macedon, NY; AOCS Ba 6a-05), and total starch (aqueous alcohol pretreatment; amyloglucosidase/α-amylase method; AOAC 996.11).

Protein, starch, fat, ash and crude fiber contents were reported as dry basis percentages (% db). Duplicate tests were conducted.

### 3.4.6  Protein Digestibility Assay

*In vitro* protein digestibility tests for sorghum flour and protein concentrates were done using the method described by Mertz and others (1984).  Samples were weighed in 200 mg portions and dispersed in 35 mL of pepsin solution (1.5 g enzyme/L of 0.1 M potassium phosphate buffer at pH 2.0).  The samples were held at $37^{o}C$ for 2 h with continuous shaking at 350 rpm using an incubator shaker (Innova 44, Pegasus Scientific Inc., Rockville, MD).  Pepsin digestion was stopped by adding 2 mL of 2 M sodium hydroxide solution.  The residue was collected after centrifugation ($4500g$, 15 min) and washed twice in 10 mL of 0.1 M potassium phosphate buffer at pH 2.0.  After the second wash, the residue was lyophilized overnight and analyzed for protein content.  Tests were performed in duplicate.  Percent *in vitro* protein digestibility, D, was computed as follows:

$$\%D = 100 \times \left[ 1 - \frac{PC_{residue} \times W_{residue}}{PC_{concentrate} \times W_{concentrate}} \right] \quad\quad\quad (2)$$

where,  PC  is the protein content (% db) and  W  is the weight (mg).

### 3.4.7  Protein solubility

Sorghum protein concentrates from selected treatments were identified for protein characterization.  Polymeric proteins were characterized using a multi-extraction procedure described by Ioerger and others (2007).  Soluble proteins (SP) were first extracted from 100 mg of ground sample using 0.5 mL of a 12.5 mM sodium borate buffer at pH 10.0 containing 2% sodium dodecyl sulfate (SDS) (w/v), with continual vortexing for 30 min. After centrifugation at 10,000$g$  for 5 min., the supernatant containing SP was removed, and the residue was sonicated (30 s at 10 W) in the same medium as above to extract the insoluble proteins (IP).  After centrifugation, the supernatant containing the IP was removed, and pellet containing residue proteins (RP) was lyophilized and analyzed for protein content.  Aliquots of the SP and IP extracts were analyzed by size exclusion high-performance liquid chromatography (SEC). The protein extracts were separated by SEC using a Biosep-3000 column (Phenomenex, Torrance, CA) with 50 mM sodium phosphate buffer at pH 7.0 containing 1% SDS as mobile phase with a flow rate of 1 mL/min.  Tests were performed in duplicate.

The SEC peak areas were summed up and %RP, %SP %IP were determined as follows:

$$\%RP = 100 \times \left( \frac{PC_{RP} \times W_{RP}}{PC_{concentrate} \times W_{concentrate}} \right) \tag{3}$$

$$\%SP = 100 \times \%SP_{area} \times \left( 1 - \frac{\%RP}{100} \right) \tag{4}$$

$$\%IP = 100 \times \%IP_{area} \times \left( 1 - \frac{\%RP}{100} \right) \tag{5}$$

where $\%SP_{area} = 100 \times \dfrac{SP_{area}}{EP_{area}}$; $\%IP_{area} = 100 \times \dfrac{IP_{area}}{EP_{area}}$; $EP_{area}$ = total area of extracted proteins

($SP_{area}$ + $IP_{area}$); $PC_{RP}$ = protein content of the residual proteins; $W_{RP}$ = weight of the residual proteins; $PC_{concentrate}$ = protein content of the concentrate; and, $W_{concentrate}$ = weight of the concentrate.

### 3.4.8 *Confocal laser scanning microscopy*

Sorghum protein concentrates from selected treatments were prepared for confocal laser scanning microscopy (CLSM) based on the procedure described by Zhao and others (2008b). Powdered samples (100 mg) were mixed with 1 mL of a weak alkaline fluorescein 5(6)-isothiocyanate (FITC) solution (0.05% w/v in 0.5 mM NaOH) and incubated in the dark at room temperature for 1 h. After which, the FITC-labeled sample was centrifuged at 13200$g$ for 4 min. The pellet was spread thinly on a glass slide and dried at room temperature in the dark. One drop of optical liquid immersion oil was added to the sample before a cover slip was placed on it, and another drop was added on top of the cover slip prior to imaging.

A laser scanning confocal microscope (Zeiss LSM 5 PASCAL, Carl Zeiss MicroImaging, Thornwood, NY) was used to image the protein microstructure of the samples. The Plan Apochromat objective (63x/1.4 oil) was used. Fluorescence emission imaging was done using 488-nm excitation; a 545 secondary dichroic was used to split the emission signals; and, a band-pass 505-530 nm filter was employed to detect FITC fluorescence. Optical sections of samples were collected every 0.7 μm and 32 slices were projected into one image that had a total thickness of 22.40 μm.

### 3.4.9  Water holding capacity

Water holding capacity (WHC) of protein concentrates from selected treatments was measured using a procedure described by Regenstein and Regenstein (1984).  A 0.5g sample of the protein concentrate was mixed with 15 mL distilled water.  The mixtures were vortexed for 5s to ensure complete dispersion and then shaken continuously  at 350 rpm and 28°C for 30 min in an incubator shaker (Innova 44, Pegasus Scientific Inc., Rockville, MD).  The samples were centrifuged at 25°C for 15 min at 4500$g$.  The weight of the water imbibed by the sediment ($W_{water\ absorbed}$) was the difference between the weight of the sample ($W_{sample} = 0.5g$) and the weight of the sediment ($W_{sediment}$).  The supernatant was freeze-dried completely for 48 h to obtain the weight of the soluble material ($W_{solubles}$).  The weight of the soluble material was then added to the weight of the water imbibed by the sediment to obtain the true weight of water absorbed by the sorghum protein concentrate.  WHC was computed as follows:

$$\text{WHC} \left( g_{water} / g_{sample} \right) = \frac{W_{water\ absorbed}}{W_{sample}} = \frac{(W_{sediment} - 0.5g) + W_{solubles}}{0.5g} \qquad (6)$$

### 3.4.10 Foaming capacity

Foaming capacity (FC) tests were also carried out on protein concentrates from selected treatments. The FC test was also done on egg white powder to serve as control.  A 0.5 g sample was mixed with 9.5 mL distilled water in a graduated test tube and shaken continuously in an incubator shaker for 1 h at 350 rpm and 28°C.  The foam volume ($V_{foam}$) was read directly and expressed as a percentage of the volume of the mixture ($V_{liquid}$) prior to shaking.

$$\%FC = 100 \times \frac{V_{foam}}{V_{liquid}} \qquad (7)$$

### 3.4.11 Statistical Analysis

Response surface analyses were conducted using SAS 9.1.3 (SAS Institute, Cary, NC). Significance was determined at $p<0.05$.  Statistical models were generated to describe protein content, digestibility and specific mechanical energy as a function of the factors that significantly explained these variables.  Optimum conditions were recommended in order to maximize protein content and digestibility.

Analysis of variance was conducted to determine significant differences in the supplementary and batch experiments, as well as in the treatments selected for protein solubility analysis.  Where differences existed, pairwise comparison was performed.

Pearson's coeficient of correlation ( r) and their significances were determined for digestibility, protein content, starch content, water holding capacity and foaming capacity using the SAS Proc Corr procedure.  The criteria defined by  Franzblau (1958) was used to describe the degree of correlation (/r/ < 0.20, negligible; /r/ = 0.20–0.40, low; /r/ = 0.40-0.60, moderate; /r/ = 0.60-0.80, marked; /r/ > 0.80, high).

## 3.5  RESULTS AND DISCUSSION

Protein content and digestibility data for sorghum protein concentrates from extrusion-enzyme liquefaction (central composite design and the supplementary experiments) are shown in Table 3.1 and Table 3.2**.**  Data from batch liquefaction experiments are shown in Table 3.3. Response surface analysis based on the data in Table 3.1 resulted in statistical models for describing specific mechanical energy (SME) input during the extrusion process, and the protein content (%PC) and digestibility (%D) of sorghum protein concentrates. These models are described in succeeding sections.  In constructing the predictive models for SME, PC and D, the principle of hierarchy in model building was maintained.  This means that if the higher order and/or interaction effect of a factor were significant, the lower order of the factor was also included in the model even if the latter was not significant.  Fig. 3.3 shows the response of SME, PC and D to each factor. Surprisingly, E2 did not have a significant role in statistical models for PC and D. This is discussed in detail later.

### 3.5.1  *Effect of MC and E1 on SME and Protein Content*

SME was significantly affected by both in-barrel moisture (MC) and enzyme level in the extruder (E1).  SME had an inverse quadratic response to increasing MC, as can be inferred from the statistical model (eq 8) and Fig. 3.3.  The statistical model for describing SME was:

$$SME\,(kJ/kg) = 3004.06 - 112.14\,MC - 97.49\,E1 + 1.15\,MC^2 + 8.54\,E1^2 \ , \ R^2 = 95.9\% \quad (8)$$

Larger differences in SME values were observed at MC levels between 14 and 32% (where the absolute value of the slope, m, was $33 \leq$/m/$\leq 75$), while smaller differences were observed at levels greater than 32% ($4 \leq$/m/$\leq 24$), thus explaining the quadratic nature o   f the response.  As MC increased, the sorghum flour melt was less viscous and did not require as

much energy to flow through the barrel and die as compared to the melt extruded at low MC. This was also reflected in the results of the supplementary experiments (Table 3.2). Without any enzyme added, SME was 1293-1509, 715 and 459-578 kJ/kg at MC 17, 32 and 50%, respectively. Several authors also reported that SME lessens with increasing MC during extrusion processing of isolated starches and flours (Koksel and others 2004; Chang and El-Dash 2003; Liu and others 2000; Govindasamy and others 1997b). Higher in-barrel MC reduces melt viscosity, which leads to lower SME (Agbisit 2007; Agbisit and others 2007).

The degree to which SME was affected by E1 was small in comparison to the effect of MC. In the model (eq 8), only 3.7% of the total variability was due to E1, while 92.2% of the total variability was due to MC. According to the response surface model, SME decreased with increasing E1 but only up to about 6% E1, and then slightly increased between 6 to 10% E1. The decline in SME with increasing E1 up to 6% can be attributed to reduced viscosity, which was brought about by the action of α-amylase on the starch fraction and/ or the presence of substantial amount of sucrose in the enzyme mix as a diluent and stabilizer. Govindasamy and others (1997b) also reported a significant reduction in SME with increasing thermostable α-amylase concentration during the extrusion-liquefaction of sago starch, i.e., from 205-472 kJ/kg in the absence of α-amylase to 76-328 kJ/kg with α-amylase. Ćurić and others (1998) found that at 55% feed moisture and greater than 20 mL thermostable α-amylase/kg dry starch, the combination of plasticization and liquefaction resulted in minimal shear, and the flow of starch melt from the extruder was in liquid form. In the current study, however, the increase in SME at E1>6% was contrary to the reasoning discussed above. This trend was predicted by the statistical model but not supported by results from the supplementary experiments (Table 3.2), which clearly showed that SME decreased as E1 increased at all three MC levels (17, 32 and 50%). The anomaly in the trend predicted by the statistical model at E1>6% could be due to deviation in SME in the case of a few CCD treatments (Table 3.1). For example, runs 3 (21-8-2) and 4 (21-8-8) were both extruded with 8% E1, but had SME of 836 and 1162 kJ/kg, respectively. This was most likely caused by variability in mass flow rate ($\dot{m}$) from 1.83 to 2.25 kg/h when E1 was greater than 6%. The hydrated sorghum flour for some treatments with high amounts of enzyme was stickier and formed clumps, which caused inconsistent flow of material from the volumetric feeder screw into the extruder. The stickiness was most probably due to high amount of sucrose in the enzyme mix.

The protein content of the concentrates was a function of both MC and E1 as described by the statistical model (eq 9). The linear component of MC explained 65.1% of the total variability, while the linear and quadratic components of E1 accounted for 4.5% and 12.4%, respectively. The statistical model relating PC with MC and E1 was:

$$\% \, PC = 129.09 - 1.70 \, MC - 10.25 \, E1 + 0.86 \, E1^2 \quad , \quad R^2 = 81.9\% \tag{9}$$

The inverse linear relationship between PC and MC was similar to the relationship of SME with MC (Fig. 3.3). As SME increased at lower MC levels, the starch in sorghum flour was degraded and gelatinized to a greater extent, making it more accessible to enzyme attack. Consequently, a purer protein concentrate was left after washing out soluble starch. Hence, high SME input (>450 kJ/kg) was needed to obtain high PC (>70% db). This was also supported by the supplementary data (Table 3.2). The only exceptions were treatments with no enzymes (0% E1 and 0% E2), in which case even high SME input did not result in concentrates with PC greater than 30%.

The quadratic response of PC to E1 was similar to the relationship of SME with E1 (Fig. 3.3). However, it was apparent that E1 also had a role independent of SME, and possibly contributed to enhanced liquefaction inside the extruder. This was supported at least partially by the supplementary extrusion-enzyme liquefaction results (Table 3.2). For example, treatment 32-5-0 had lower SME than 32-0-0 (261 kJ/kg and 715 kJ/kg, respectively) but higher PC (56% db and 17% db, respectively), indicating the role of α-amylase in bringing about liquefaction during extrusion. Previous studies also reported that higher E1, especially at higher MC, improved hydrolysis during extrusion-enzyme liquefaction of corn starch (Solihin and others 2007; Chouvel and others 1983) and sago starch (Govindasamy and others 1997b). These findings and supplementary data from the current study contradict the statistical model (eq 9), which predicted an increase in PC only at E1 > 6%. It is clear that future research needs to focus on the impact of enzyme on in-barrel liquefaction, especially the role of reduced SME brought about by enzyme addition.

### 3.5.2 Effect of E2 on PC

Although the response surface model (eq 9) indicated that PC was not a function of post-extrusion enzyme concentration (E2), supplementary data (Table 3.2) indicated that addition of E2 led to enhancement of PC when E1 was absent. To illustrate, sorghum flour extruded at 17-

0-0 yielded only 26% PC, but post-extrusion liquefaction with 2% enzyme (17-0-2) increased PC to 71% db. Similar effect was seen between 50-0-0 and 50-0-10 treatments.

The importance of E2 was further validated in the results of control batch liquefaction experiments (Table 3.3). Without enzyme, the PC of the concentrate was only 35% db, but adding the enzyme yielded concentrates with PC ranging from 72 to 75% db. The higher PC obtained with adding the enzyme post-extrusion rather than in the extruder was due to both the high moisture condition and longer liquefaction time during post-extrusion batch liquefaction. Additionally, the reduction of SME due to in-barrel enzyme addition led to lower PC. For example, 32-5-0 had 56% db PC (Table 3.2), but 32-0-5 had 82% db PC (run 11, Table 3.1). Likewise, 50-10-0 had 24% db PC, while 50-0-10 had 78% db PC. As shown by several authors, starch liquefaction is promoted by the high mobility of enzymes and more extensive starch gelatinization at high moisture conditions (Baks and others 2008; Govindasamy and others 1997b; Grafelman and Meagher 1995; Komolprasert and Ofoli 1991; Lee and Kim 1990; Chouvel and others 1983). Increasing post-extrusion liquefaction time either by attaching a static mixer (Grafelman and Meagher 1995) or a batch reactor (Baks and others 2008) at the end of the extruder were effective in increasing corn and wheat starch degradation, respectively.

In the presence of E1, however, it appeared that the role of E2 was negligible or at best only secondary during post-extrusion batch liquefaction. This could be the reason for E2 not appearing in the response surface model for PC. The underlying hypothesis is that the activity of E1 persisted through post-extrusion batch liquefaction, minimizing the effect of E2. For treatments requiring post-extrusion-batch liquefaction, enzyme inactivation was not carried out immediately after extrusion but it was done only after liquefaction with E2. Since E1 was most probably still viable post-extrusion, the addition of E2, even at higher levels, did not show large increments in PC. For instance, treatment 17-0-2 yielded 71% db PC, while 17-0-5 yielded 72% protein (Table 3.2). The control batch liquefaction treatments (Table 3.3) also showed that the addition of α-amylase at least doubled the protein content obtained but that raising the concentration of the enzyme did not always raise the protein levels. It is evident that addition of E2 beyond 2% was no longer beneficial. Although our results showed that increasing enzyme concentration did not lead to significantly greater sorghum starch degradation, Aggarwal and others (2001) and Zhao and others (2008c) have shown otherwise in the batch liquefaction of sorghum flour. More extensive starch liquefaction resulted when Aggarwal and others (2001)

increased α-amylase concentration from 0.01 to 0.3%, v/w and when Zhao and others (2008c) increased Liquozyme SC DS (the same enzyme used in our study) dosage from 0.04 to 0.84% fwb.   The contrasting results that we obtained may be because even the lowest enzyme dosage used in our study was more than double those used by Aggarwal and others (2001) and Zhao and others (2008c).

### 3.5.3 *Effect of batch- and extrusion-enzyme liquefaction on in vitro protein digestibility*

The statistical model for *in vitro* protein digestibility is shown below.

$$\% D = -22.69 + 4.47\,MC + 3.21\,E1 - 0.06\,MC^2 - 0.10\,MC \times E1, \quad R^2 = 82.5\% \quad (10)$$

The linear and quadratic components of MC explained 74.4% of the total variability and the interaction term MC x E1 accounted for 8.1%. Although variability due to the linear component E1 was practically nil (0.001%), it was incorporated in the model in order to satisfy the principle of hierarchy.

Raw, decorticated sorghum flour digestibility obtained in this study (76%) was similar to those reported in literature (65 to 80%) (Nunes and others 2004; Duodu and others 2002; Weaver and others 1998; Oria and others 1995; Rom and others 1992; Hamaker and others 1986), and higher than either extruded (<66%) or batch liquefied (50 to 56%) protein concentrates. Additionally, the digestibility values of batch liquefied samples, which were cooked in excess water and boiled for 20 min to inactivate enzymes, were close to the value obtained by Weaver and others (1998) for sorghum flour boiled in water (57%).   Other authors also reported reduction in *in vitro* protein digestibility as a result of cooking sorghum flour in excess water (Duodu and others 2002; Hamaker and others 1986).

Sorghum protein bodies and starch granules are tightly bound into the protein matrix (Wong and others 2009; Rooney and Pflugfelder 1986; Taylor and others 1984;), hindering enzyme accessibility and resulting in poor digestibility.   The disruption of this spatial arrangement in sorghum proteins and starch due to shear mechanical forces during extrusion improves digestibility.   Thus, increasing SME would have resulted in more degraded and digestible sorghum proteins than those of raw sorghum flour.   Accompanying protein disaggregation is the unfolding of protein molecules, exposition of hydrophobic and hydrophilic amino acid residues, and breakage/ reformation of disulfide and peptide bonds (Camire 1991).

Indeed, several studies have shown that low moisture, high temperature extrusion raises *in vitro* protein digestibility of raw sorghum flour from about 60% to at least 75% (Hamaker and others 1994; Dahlin and Lorenz 1993; Fapojuwo and others 1987; Mertz and others 1984; MacLean and others 1983). However, our results indicate otherwise. This contradiction was probably brought about by re-aggregation of protein fragments during the boiling step of enzyme inactivation. With the large number of hydrophobic amino acid residues in sorghum proteins (Belton and others 2006), it is highly probable for these to interact and form protein aggregates, as well as for intermolecular disulfide bridges to reform. This explains why the highest *in vitro* protein digestibility attained from extrusion-enzyme liquefaction was only 66% in spite of the high SME achieved in this study. In spite of which, digestibility of extrusion liquefied treatments were significantly better than those batch liquefied.

The addition of α-amylase (37$^o$C, 1h) was shown to increase *in vitro* protein digestibility from 37% (cooked, no α-amylase) to 42% (cooked, with α-amylase) of cooked, white, whole grain sorghum flour, and from 39% (cooked, no α-amylase) to 44% (cooked, with α-amylase) of cooked, white, decorticated sorghum flour (Duodu and others 2002). Hence, raising E2 concentration was expected to increase *in vitro* protein digestibility. However, results of the optimization (Fig. 3.3) and supplementary (Table 3.2) experiments did not reflect this. Although slightly higher %D was observed with increasing enzyme concentration in the batch experiments (Table 3.3), this was not significant. Possibly, the effects of increasing E2 were so slight that protein re-aggregration overrode any observable increase in digestibility brought about by raising E2. Furthermore, as discussed in the preceding section on the effects of E2 on PC, even the lowest levels of E2 used in this study were so much higher than those used in previous studies (Zhao and others 2008c; Aggarwal and others 2001;) that probably any incremental improvements in digestibility were not significant.

### 3.5.4  *Optimum processing conditions*

The results of the study showed that extrusion-liquefaction was more advantageous than batch liquefaction in producing pure and digestible sorghum protein concentrates under optimal conditions even though it failed to do so under a number of processing conditions. Numerical optimization conducted using SAS ADX showed that the optimum set of conditions to concurrently obtain the highest protein content (75% db) and digestibility (65%) is 32 % MC,

0% E1 and 2.5% E2 (SME = 593 kJ/kg). This processing condition can be used in future work to achieve a good balance between protein content and digestibility. The protein content obtained under these optimum conditions is higher than that from batch liquefaction. Several other processing conditions for extrusion-enzyme liquefaction also led to higher protein content than batch liquefaction. For example, at 17% MC, 5% E1 and 5% E2, sorghum protein concentrates produced by extrusion-liquefaction have higher protein content (79% db) (Table 2). Additionally, 82% db protein content can be achieved by extruding at 32% MC, 0% E1 and 5% E2 (Table 1). However, several process conditions for extrusion-enzyme liquefaction led to lower protein content and protein digestibility than batch liquefaction. This is expected as any optimization study would lead to results on either side of the optimum. Apart from obtaining sorghum protein concentrates with higher protein content than other methods, advantages of the extrusion-enzyme liquefaction process include lower process moisture content (≤32%), and potentially low enzyme concentration, shorter processing time, and higher throughput or production capacity.

### 3.5.5  *Protein solubility*

Protein characterization was done for selected treatments to determine the extent of protein disaggregation that resulted from varying process conditions. Sorghum polymeric proteins were fractionated based on their solubility in different reagents based on the procedure by Ioerger and others (2007). Proteins soluble in SDS without sonication (SP) correspond to the albumin, globulin and kafirin-1 fraction of the Landry-Moureaux (L-M) fractionation procedure (Hamaker and others 1986) and are composed primarily of monomeric kafirins; proteins extractable in SDS only after sonication (IP) correspond primarily to the L-M kafirin-2 fraction (crosslinked kafirins) and contain some glutelin; and, the remaining residue protein fraction (RP) is composed of the L-M glutelin-like, true glutelin and unextractable proteins (non-kafirins). The RP fraction is highly crosslinked and nonextractable by SDS and sonication, thus is less digestible than the SP and IP fractions. The percentages of the SP, IP and RP fractions of the selected treatments were plotted in Fig. 3.4 and *in vitro* protein digestibility values were also indicated. The reduction in digestibility that accompanied extrusion and batch processing was supported by the rather large percentage of RP for all processed samples (RP was 68 to 93% db).

However, these results failed to reveal differences between extrusion- and batch-liquefied samples and also did not show a direct relationship between digestibility and protein solubility.

The absence of a straight forward relationship between protein digestibility and solubility in our work is possibly due to differences in the digestibility of the RP fraction, which was the predominant component of the concentrates produced by either batch or extrusion liquefaction. Based on the study of Hamaker and others (1994), the underlying mechanism behind protein changes during extrusion was not entirely one of complete protein disaggregation and that the RP fraction has a substantial amount of digestible proteins. In their study, extrusion of decorticated sorghum flour with less than 20% MC at $177^{o}$C resulted in an increase in protein digestibility (in comparison to raw sorghum flour) in spite of the increase in nonextractable proteins (part of the RP fraction). Hamaker and others (1994) inferred that improvement in protein digestibility was a result of the increase in kafirin-1 (corresponds to SP fraction) and the reduction in kafirin-2 (crosslinked kafirin; corresponds to IP fraction) and true glutelin (crosslinked glutelin; part of the RP fraction). In contrast, boiling sorghum reduces protein digestibility, which is related to protein polymerization (Ezeogu and others 2008; Nunes and others 2004; Duodu and others 2002; Rom and others 1992; Hamaker and others 1986) and to a shift towards increasing amounts of crosslinked glutelins and nonextractable proteins at the expense of the albumin, globulin, kafirin and glutelin-like protein fractions (Wong and others 2009; Hamaker and others 1986). Confocal laser scanning microscopy was a better tool in explaining the underlying differences in protein digestibility, and the results of which are discussed in the next section.

### 3.5.6 *Confocal Laser Scanning Micrographs*

CLSM micrographs (Fig. 3.5) showed the changes in protein aggregation/ disaggregation during processing. Prior to extrusion, the protein bodies were embedded in the glutelin protein matrix in a distinct honeycomb fashion and raw starch granules had distinct shape. During extrusion, the honeycomb arrangement of the protein bodies was lost and proteins aggregated, forming a matted protein matrix. Starch was gelatinized and most granules lost their shape. Some "starch ghosts", which were probably starch granules that were not completely degraded or gelatinized, remained embedded in the protein matrix.

The extent of protein aggregation/ disaggregation was also visible in the micrographs, revealing underlying differences in digestibility. Sections of selected treatments representing a

range of protein digestibilities are presented in Fig. 3.6. The sample with the lowest digestibility (Fig. 3.6A) had a highly aggregated protein matrix. In contrast, the most digestible sample (Fig. 3.6E) had a protein matrix made up of thin discontinuous protein fibrils. These discontinuities made the protein easily accessible to pepsin and provided a large surface area for digestion. In general, the proteins appeared less aggregated and more discontinuous with increasing protein digestibility. The batch liquefied treatment (Fig. 3.6F) had a thick and slightly discontinuous protein aggregate. The extruded treatment with similar digestibility to the batch liquefied treatment (Fig. 3.6C) had larger pieces of protein aggregates than this batch liquefied sample, but these large protein pieces were not as thickly aggregated as those of the batch liquefied sample. Protein structural changes such as the extensive aggregation of the glutelin protein matrix, observed as formation of a thick layer of webbed protein enveloping kafirins and starch, that occur during wet cooking has been identified as one of the reasons for reduced protein digestibility (Hamaker and Bugusu 2003; Oria and others 1995; Chandrashekar and Kirleis 1986). The relationship between protein digestibility and structural changes evident in our CLSM micrograph of the batch liquefied treatment are consistent with those seen in previous studies.

### 3.5.7 CLSM and Protein Solubility

The micrographs supported the observed changes in protein solubility (Fig. 3.4) for treatments 32-0-5 (Fig. 3.6C) and 32-5-5 (Fig. 3.6E). Treatment 32-0-5 had a higher RP content (92.65% db) and lower SP content (2.45% db) than treatment 32-5-5 (89.68 and 5.60% db RP and SP, respectively). The extensive re-aggregation of proteins shown in the CLSM micrographs explained why fewer proteins could be extracted with SDS from 32-0-5. These heavily aggregated protein pieces were so strongly crosslinked that even an added sonication step was insufficient in breaking these. The treatment subjected to greater shear (32-0-5; SME=687 kJ/kg) had possibly more exposed hydrophobic amino acid and sulfhydryl reactive sites than the treatment with lower shear (32-5-5; SME=329 kJ/kg). Subsequent boiling in the enzyme inactivation step precipitated the union of these reactive sites, which led to the re-aggregation of proteins. A greater number of exposed reactive sites could lead to a larger number of crosslinks, possibly making proteins resistant to reduction and digestion. This could also be reflected in protein solubility, i.e., more extensive protein crosslinking would result in a

higher RP percentage.  Hence, larger protein aggregates and a higher RP percentage were observed in treatments subjected to higher SME.  Conversely, treatments with lower SME had minimal protein re-aggregation, making them more soluble (higher SP fraction) and digestible.

### 3.5.8  *Water holding and foaming capacities*

Water holding capacity (WHC) and foaming capacity (FC) were conducted for the treatments selected for SEC analysis in order to evaluate functionality.   WHC of the extruded sorghum protein concentrates ranged from 2.21 to 3.79 g water/g sample.  Untreated sorghum flour and batch liquefied sorghum flour had WHC of 1.84 and 4.00 g water/ g sample, respectively.  Fig. 3.7 shows correlations amongst the functional and chemical properties of the sorghum flour and sorghum protein concentrates.  WHC was not related to protein and starch contents and to digestibility (Fig. 3.7A).  The absence of a correlation between WHC and these components suggests that molecular changes in protein and starch could have affected WHC.  As indicated in the CLSM micrographs, extrusion significantly affected protein solubility and aggregation, which most probably affected WHC.  The present work, however, does not show a single trend that explains differences in WHC.  The underlying relationship between WHC and the molecular changes in extruded sorghum protein and starch can be explored in future work.

Sorghum protein concentrates had either no or poor foaming capacities.  Sorghum protein concentrate FC were 60 to 350%, while egg white FC was 750%.  FC was markedly related to digestibility (r = 0.69, Fig. 3.7B).  A more digestible protein concentrate has small molecular weight proteins, which are responsible for large and stable foams.  Kunst (2003) noted that protein hydrolysates used in food foams have molecular weights ranging from 2 to 5 kDa.  On the other hand, egg white, which forms good foams has a molecular weight of 14.3 kDa.  The foams from sorghum protein concentrates were not stable because they were made of large bubbles, which readily coalesced.  In contrast, egg white foam was made of several small bubbles that did not coalesce. The low foaming capacities of the sorghum protein concentrates is most probably because the proteins have large molecular weights.

## 3.6  CONCLUSIONS

In conclusion, extrusion-enzyme liquefaction of sorghum flour yielded protein concentrates with protein contents (PC) and digestibility (D) higher than those of the concentrates obtained by batch liquefaction at optimal process conditions.  However, as typical

of optimization studies, extrusion-enzyme liquefaction conditions at either side of the optimum led to lower protein content and digestibility than batch liquefaction. Extrusion in-barrel moisture (MC) had the greatest influence on specific mechanical energy (SME), PC and D. Reducing MC raised SME, which led to higher PC. Enzyme added during extrusion (E1) had a significant impact in raising PC when there was no enzyme added post-extrusion (E2). While reducing E1 brought about an increase in SME, the statistical model suggested that the role of E1 in raising PC and D was small. The presence of E2 becomes crucial in the absence of E1 because starch liquefaction occurred solely during batch liquefaction. However, raising E2 concentration did not result in significantly higher protein contents. Statistical results suggested that the optimum set of conditions for producing a sorghum protein concentrate that had both high protein content and high digestibility was 32% MC, no enzyme added during extrusion, and 2.5% E2. Protein solubility did not distinguish differences in digestibility between batch and extrusion liquefied treatments because both methods of liquefaction had large residue protein fractions and minimal differences in soluble and insoluble protein fractions. CLSM provided better explanation for digestibility based on visual images of the extent of protein disaggregation. The protein concentrate with highest digestibility had a more discontinuous protein structure and was made up of thin protein fibrils, while the least digestible one had large areas of thickly aggregated proteins. Extrusion-enzyme liquefaction has the advantage of producing highly pure and digestible sorghum protein concentrates using lower process moisture, and potentially less enzyme, reduced processing time, and higher throughput or production capacity because it hastens starch liquefaction and removal. Future work includes scale-up of the extrusion-liquefaction process as well as conducting a more extensive study of the factors affecting it.

## 3.7 ACKNOWLEDGEMENTS

## 3.8   REFERENCES

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Barrel Temperature (°C): Feed input | 40 | 50 | 60 | 70 | 70 | 70

Element positions: 0ᵃ 1 2 3 4 5 6 7 8 9 10 11
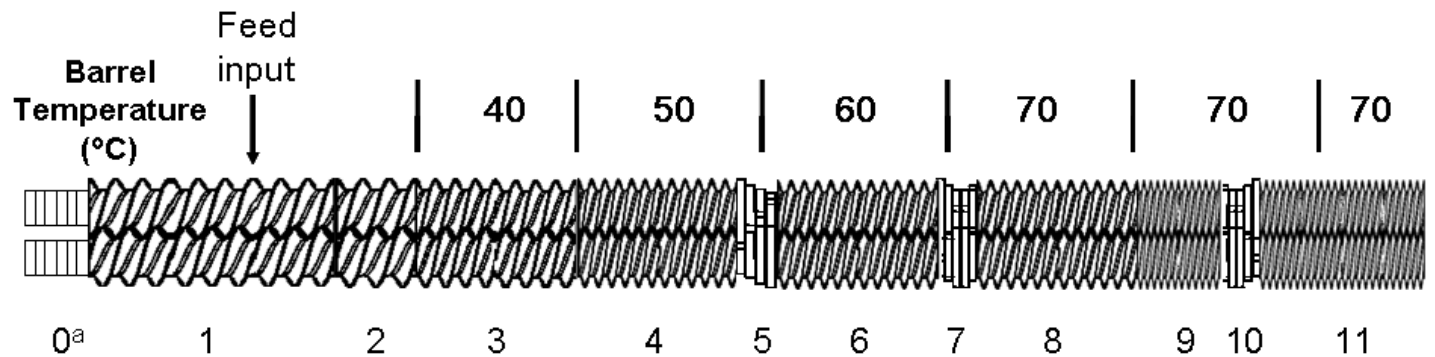
ᵃElement Numbers: 0 = spacers*; 1 = SE, 30-90; 2 =SE, 30-30; 3 = SE, 20-60; 4 = SE, 15-60; 5 = KB, 4-5-20-30R; 6 = SE, 15-60; 7 = KB, 5-4-20-45F; 8 = SE, 15-60; 9 = SE, 10-30; 10 = KB, 5-4-20-45R; 11 = SE, 10-60

* element 0: 6 spacers; 4.75 mm length per spacer ; 28.5 total length

Legend:
SE=screw element
Numbers:
1st – length of flight (mm)
2nd - total element length (mm)

KB = kneading block
Letters: F- Forward, R- Reverse
Numbers:
1st- number of disks
2nd- length of disks (mm)
3rd- total element length (mm)
4th-angle of disks

Fig. 3.1 Screw configuration for extrusion-enzyme liquefaction experiments.
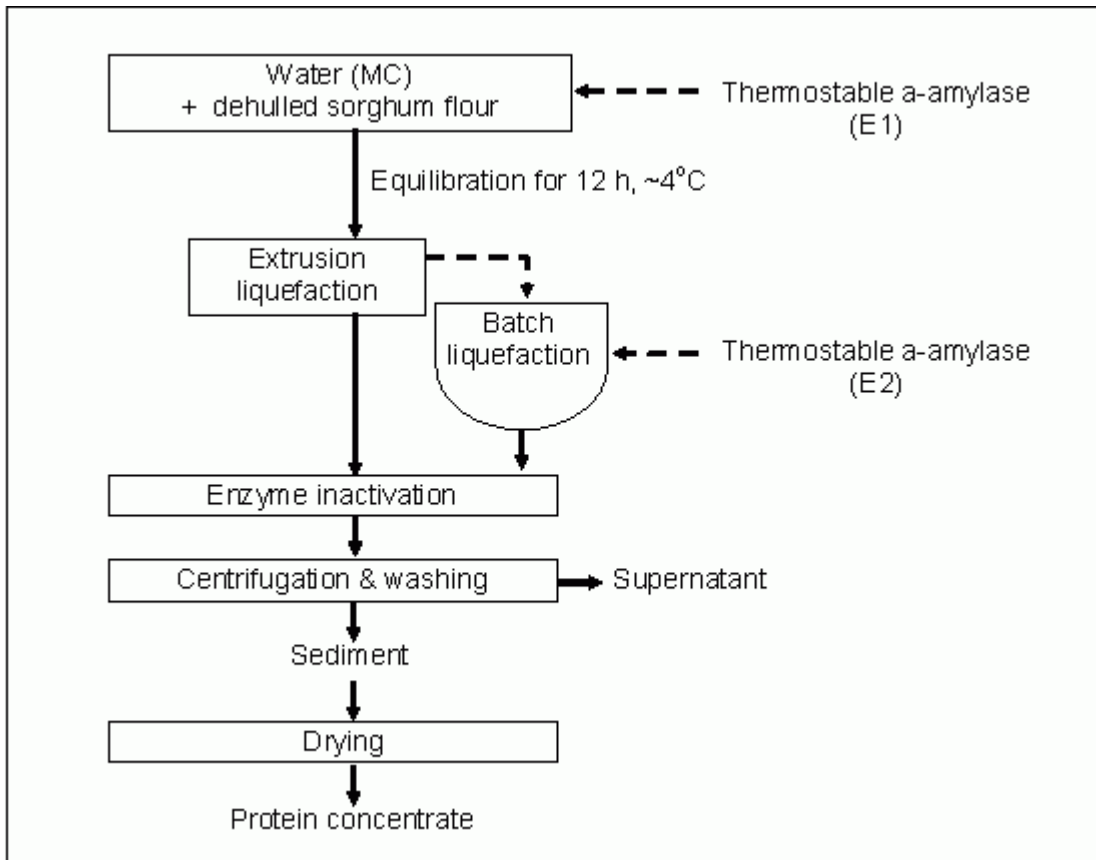
**Fig. 3.2 Extrusion-enzyme liquefaction process**
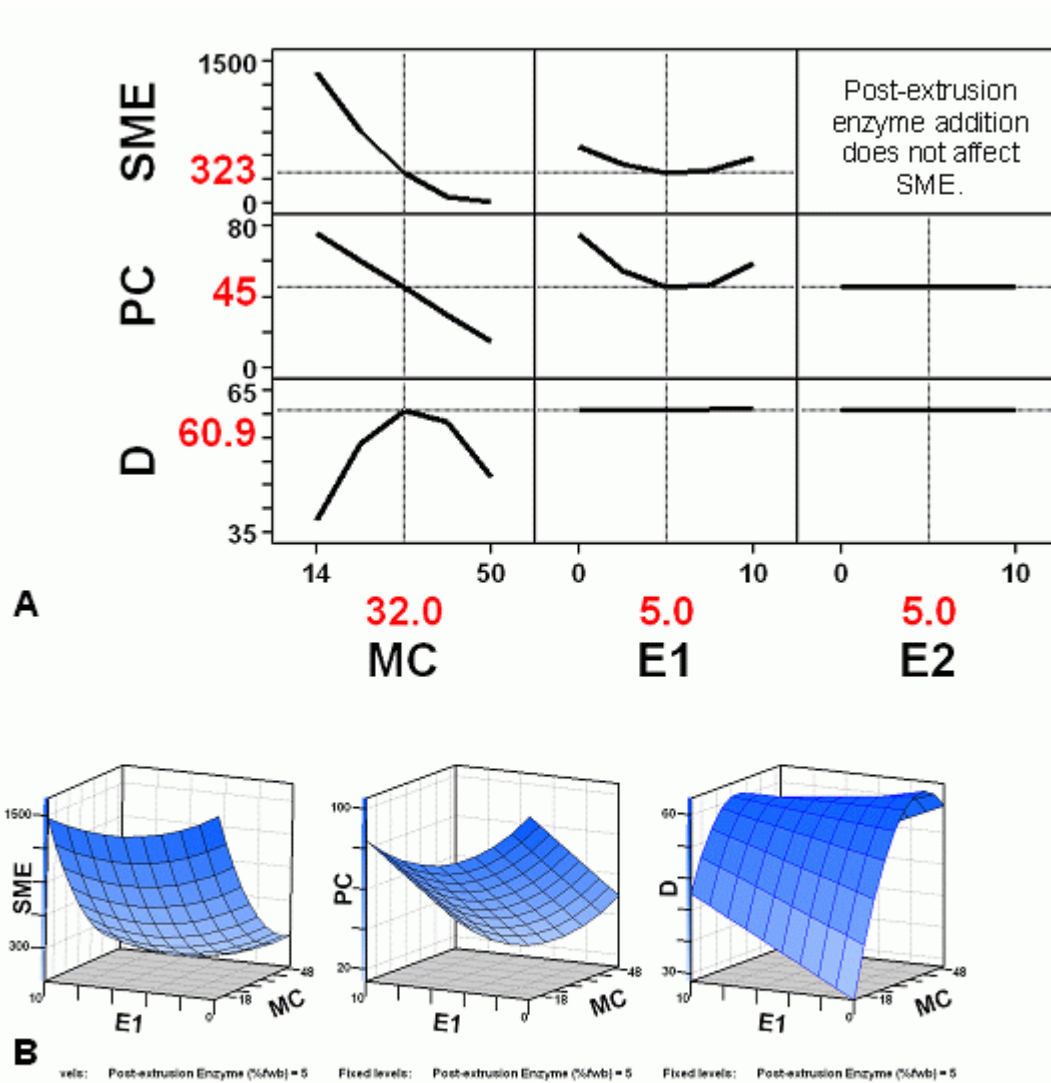
Legend: **MC**, % flour weight basis (fwb) In-barrel moisture content; **E1**, % fwb enzyme concentration during extrusion; **E2**, % fwb post-extrusion enzyme concentration; **SME**, kJ/kg, specific mechanical energy; **PC**, % protein content, dry basis; **D**, % *in vitro* protein digestibility

**Fig. 3.3 Protein content, digestibility and specific mechanical energy as a functions of in-barrel moisture content and enzyme concentration during extrusion, 5% fwb post-extrusion enzyme addition.**
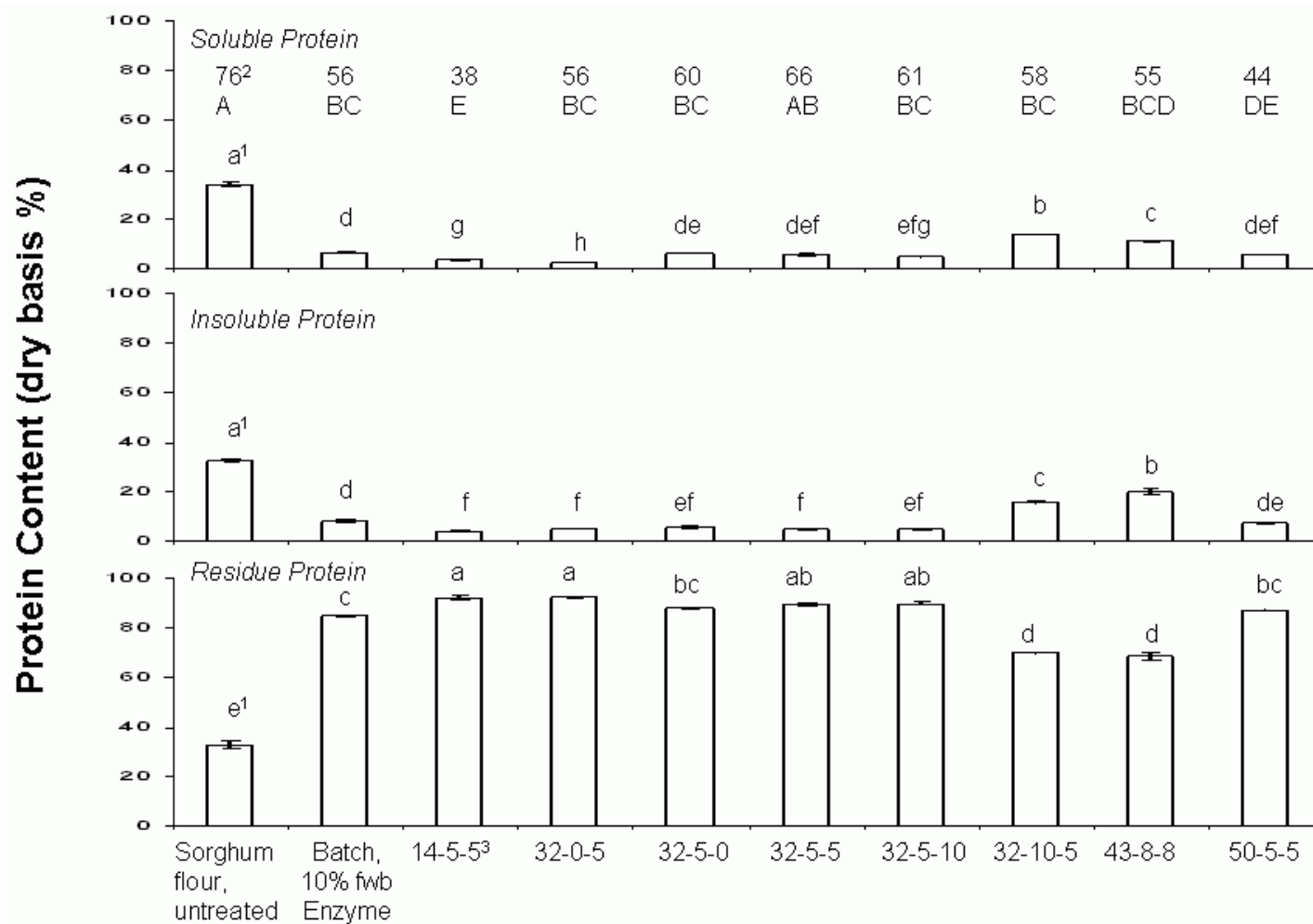
84

**Fig. 3.4 Soluble, insoluble and residue sorghum proteins in relation to *in vitro* protein digestibility.**
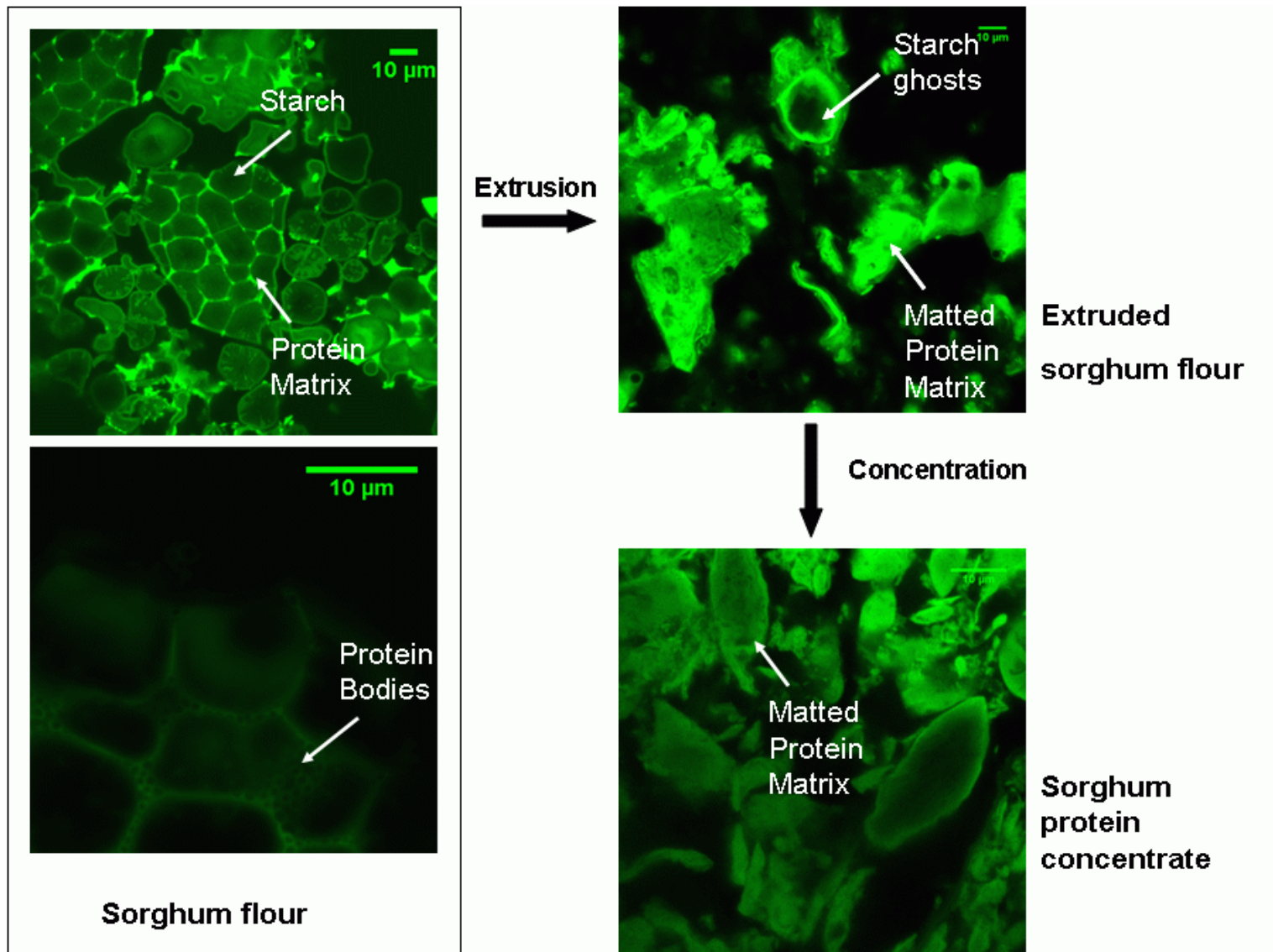
**Fig. 3.5 Progression of protein disaggregation and starch degradation depicted using confocal laser scanning microscopy.**
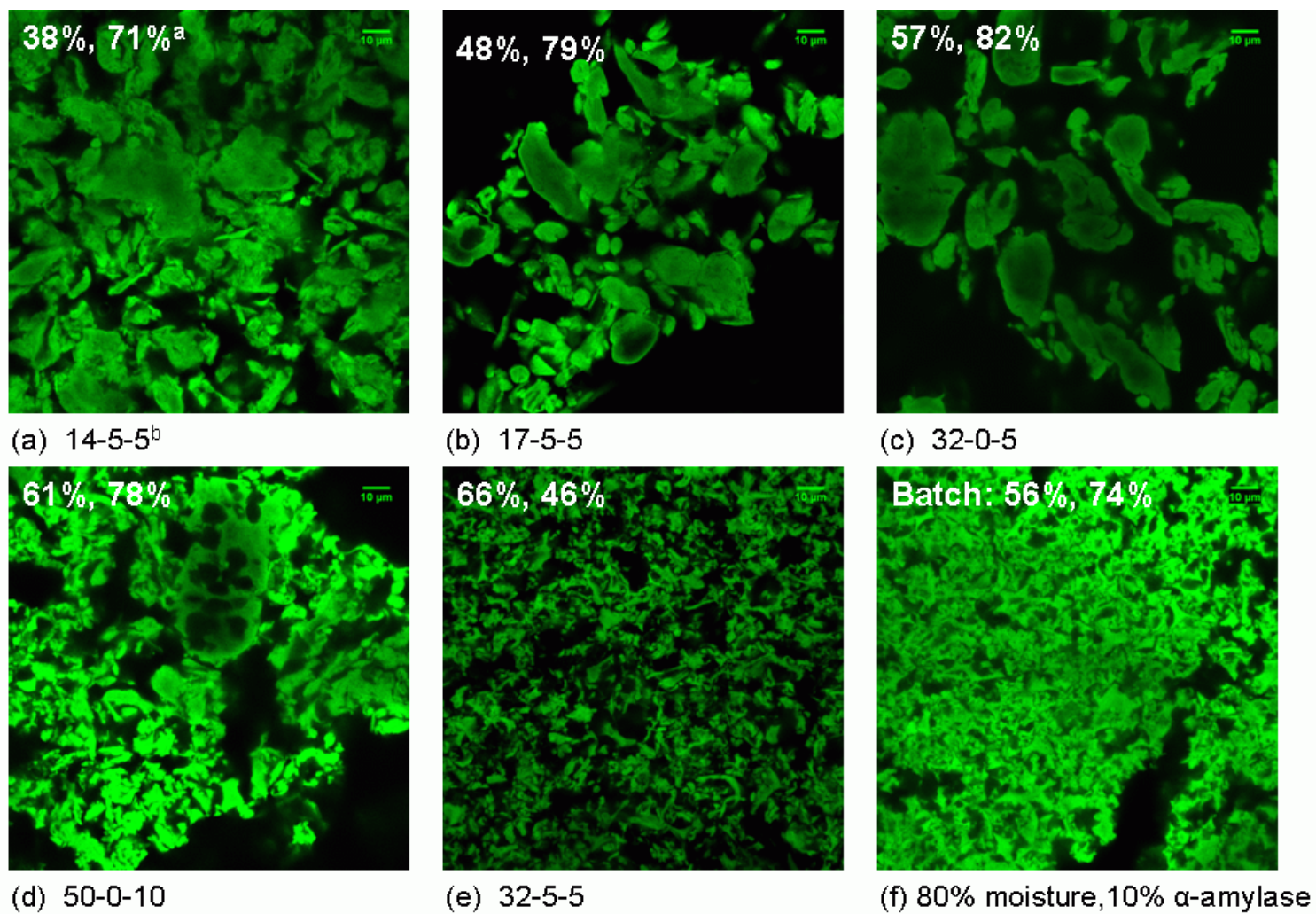
Fig. 3.6 Depiction of sorghum protein aggregation/ disaggregation by confocal laser scanning microscopy in relationship to its *in vitro* protein digestibility.
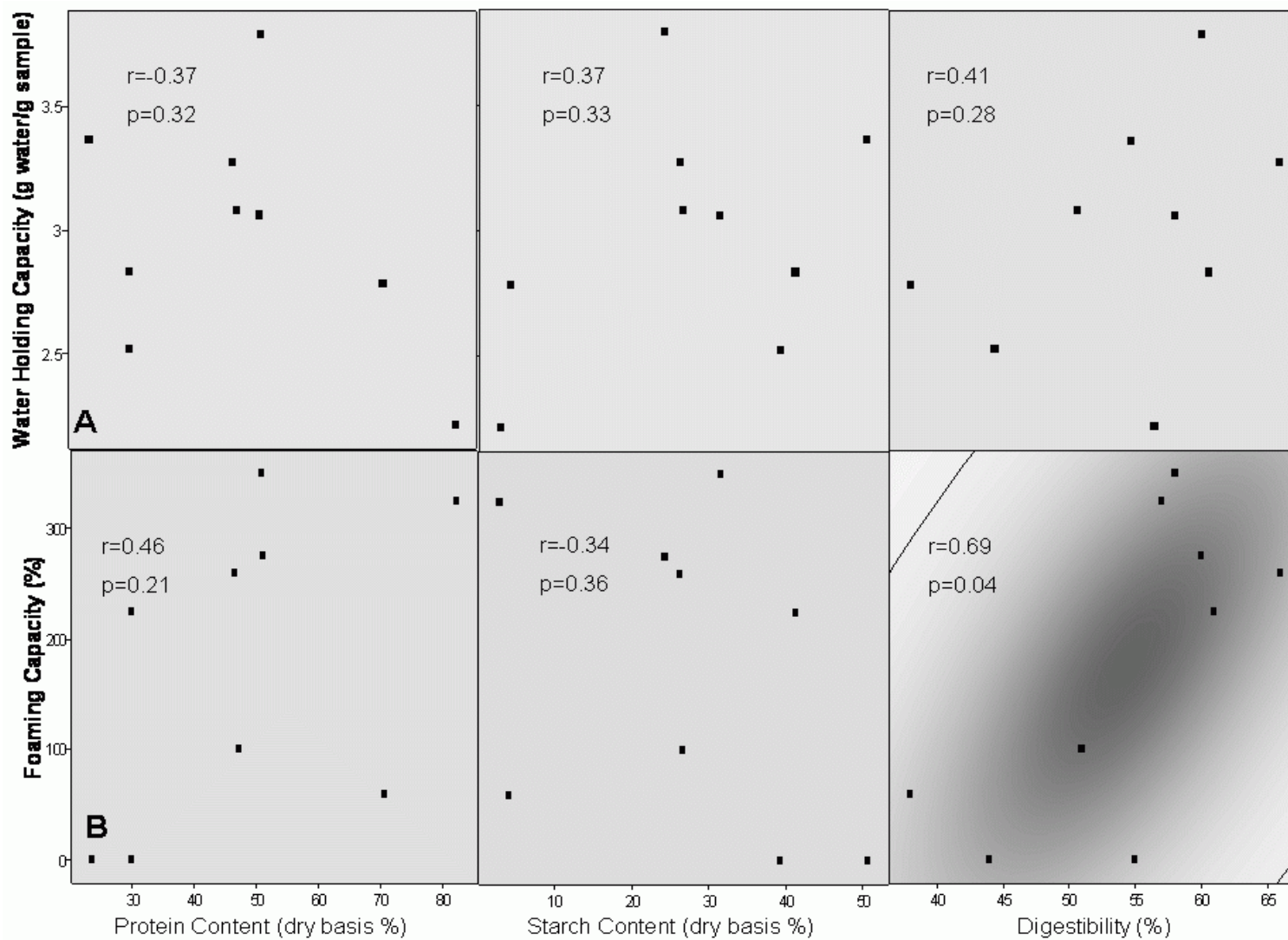
**Fig. 3.7 Water holding (A) and foaming (B) capacities of sorghum protein concentrates.**

Notes: (1) r is the correlation constant; (Franzblau criteria states that /r/ < 0.20, negligible; /r/ = 0.20–0.40, low; /r/ = 0.40-0.60, moderate; /r/ = 0.60-0.80, marked; /r/ > 0.80, high) ; (2) p is the level of significance, i.e., p<0.05 denotes a significant correlation.

**Table 3.1 Central Composite Design**

| RUN | In-barrel moisture content, % fwb (MC) | Enzyme in the extruder, % fwb (E1) | Post-extrusion enzyme, % fwb (E2) | Specific Mechanical Energy, kJ/kg (SME) | Protein Content, % db (PC) | Digestibility, % (D) |
|---|---|---|---|---|---|---|
| 1 | 21 | 2 | 2 | 999 | 72.22 | 54.44 |
| 2 | 21 | 2 | 8 | 851 | 77.75 | 42.81 |
| 3 | 21 | 8 | 2 | 836 | 71.82 | 54.15 |
| 4 | 21 | 8 | 8 | 1162 | 79.73 | 54.74 |
| 5 | 43 | 2 | 2 | 141 | 33.36 | 65.18 |
| 6 | 43 | 2 | 8 | 129 | 34.49 | 57.81 |
| 7 | 43 | 8 | 2 | 89 | 30.49 | 54.27 |
| 8 | 43 | 8 | 8 | 55 | 23.55 | 54.88 |
| 9 | 14 | 5 | 5 | 1336 | 70.62 | 38.05 |
| 10 | 50 | 5 | 5 | 51 | 30.02 | 44.47 |
| 11 | 32 | 0 | 5 | 687 | 82.38 | 56.66 |
| 12 | 32 | 10 | 5 | 379 | 50.98 | 58.2 |
| 13 | 32 | 5 | 0 | 332 | 51.05 | 60.28 |
| 14 | 32 | 5 | 10 | 266 | 29.94 | 60.81 |
| 15 | 32 | 5 | 5 | 396 | 43.35 | 59.8 |
| 16 | 32 | 5 | 5 | 293 | 51.26 | 61.53 |
| 17 | 32 | 5 | 5 | 310 | 54.76 | 61.46 |
| 18 | 32 | 5 | 5 | 329 | 46.57 | 66.19 |
| 19 | 32 | 5 | 5 | 311 | 36.8 | 63.14 |
| 20 | 32 | 5 | 5 | 340 | 37.06 | 57.68 |

**Table 3.2 Supplementary extrusion-liquefaction experiments**

| In-barrel moisture content, % fwb (MC) | Enzyme in the extruder, % fwb (E1) | Post-extrusion enzyme, % fwb (E2) | Specific Mechanical Energy, kJ/kg (SME) | Protein Content, % db (PC)[a,b] | Digestibility, % (D)[a,b] |
|---|---|---|---|---|---|
| 17 | 0 | 0 | 1509 | 25.78 (0.21)g | 47.60 (1.81)c |
| 17 | 0 | 2 | 1410 | 71.50 (0.06)d | 44.69 (1.51)cd |
| 17 | 0 | 5 | 1293 | 72.26 (0.06)c | 41.09 (0.14)d |
| 17 | 5 | 5 | 1216 | 78.85 (0.13)a | 47.50 (2.55)c |
| 32 | 0 | 0 | 715 | 17.35 (0.01)i | 46.90 (1.20)c |
| 32 | 5 | 0 | 261 | 55.80 (0.06)e | 54.55 (3.04)b |
| 32 | 10 | 0 | 257 | 35.43 (0.00)f | 54.87 (0.01)b |
| 50 | 0 | 0 | 578 | 10.81 (0.01)j | 46.53 (4.83)c |
| 50 | 0 | 10 | 459 | 78.17 (0.09)b | 60.78 (0.52)a |
| 50 | 10 | 0 | 0 | 23.84 (0.05)h | 56.11 (1.79)ab |

[a]Average of two replicates (standard deviation)
[b]Numbers with different letters within each column are significantly different ($p < 0.05$)

**Table 3.3 Protein Content and Digestibility**

**of Untreated and Batch Liquefied Sorghum Flour**

| Moisture content, % fwb | Enzyme concentration, % fwb | Protein content (% db)[a,b] | Digestibility (%)[a,b] |
|---|---|---|---|
| Sorghum flour, untreated | No treatment | 9.68 (0.03)f | 76.35 (0.96)a |
| 80 | 0 | 35.33 (0.04)e | 51.70 (2.970)b |
| 80 | 2 | 75.28 (0.12) a | 50.24 (2.54)b |
| 80 | 5 | 72.78 (0.04)c | 52.63 (1.67)b |
| 80 | 10 | 73.50 (0.13)b | 55.97 (3.41)b |
| 80 | 15 | 72.11 (0.09)d | 53.94 (2.81)b |

[a]Average of two replicates (standard deviation)
[b]Numbers with different letters within each column are significantly different ($p < 0.05$)

# Chapter 4 - Pilot-Scale Processing of Sorghum Protein Concentrates Using Extrusion-Enzyme Liquefaction[1]

## 4.1 ABSTRACT

Factors affecting the pilot-scale production of sorghum protein concentrates using extrusion-enzyme liquefaction were studied. This included studies of effects of in-barrel moisture content (17 and 32% dry basis, db), extruder screw speed (200, 300 and 400 rpm) and post-extrusion enzyme concentration (0.5, 1.5 and 2.5% flour weight basis, fwb) on specific mechanical energy, protein content (measure of purity), and protein digestibility before and after liquefaction. Raw and batch liquefied sorghum flours served as control. Sorghum protein concentrates produced by extrusion-enzyme liquefaction had higher protein purity (protein content ranging from 72 to 80% db) and *in vitro* protein digestibility (62 to 70%) than did either raw (10% db, 58%) or batch liquefied sorghum flour (70% db, 57%). Extrusion pre-treatment of sorghum flour simultaneously broke the tight association between protein and starch, disrupted sorghum protein networks, disorganized the compact starch crystalline structure, and gelatinized starch. Altogether, these reactions increased the speed and improved the efficiency of downstream liquefaction and protein concentration processes. Extrusion-enzyme liquefaction is a high throughput method for producing sorghum protein concentrates with the potential for commercial scale-up. Sorghum is safe for consumption by celiac patients, and sorghum protein concentrate can improve the nutritional and functional qualities of gluten-free foods.

## 4.2 NOMENCLATURE

E- Enzyme Concentration

EDTA- Ethylene Diamine Tetraacetic

EP – Extracted Proteins

IP – Insoluble Proteins

MC – In-barrel Moisture Content

PC – Protein Content

$D_{after}$- *In vitro* Protein Digestibility after liquefaction

$D_{before}$- *In vitro* Protein Digestibility before liquefaction

SME – Specific Mechanical Energy

SP – Soluble Proteins

SPC – Sorghum protein concentrate

RP – Residual Proteins

## 4.3 INTRODUCTION

Sorghum (*Sorghum bicolor*) grain has an average protein content of 11% (Lasztity 1996) and its proteins are broadly classified as prolamins (kafirin) and non-prolamins (albumin, globulin, glutelin). Kafirins, which make up most of the sorghum proteins (77 to 82% of the endosperm proteins), exist as rigid protein bodies that are heavily embedded in a glutelin protein matrix (Taylor and others 1984). Because of this morphological feature, kafirins are very difficult both to disrupt and digest (de Mesa-Stonestreet and others 2010; Belton and others 2006; Duodu and others 2003). Kafirins also have poor viscoelastic properties due to the extensive disulfide crosslinking of kafirin monomers (Oom and others 2008). It is known that sorghum protein digestibility is further reduced by wet cooking, wherein protein crosslinking is enhanced (Nunes and others 2005; Duodu and others 2002b; Rom and others 1992; Hamaker and others 1986). *In vitro* protein digestibility of raw, decorticated sorghum flour is 65 to 80%, while that of cooked, decorticated sorghum flour is 40-58% (Duodu and others 2002a; Weaver and others 1998; Oria and others 1995). Likewise, raw sorghum starch digestibility is hampered by the existence of kafirin protein bodies (Chandrashekar and Kirleis 1988; Hamaker and others 1987;) and the glutelin protein matrix that tightly envelopes starch granules (Choi and others 2008; Rooney and Serna-Saldivar 2000; Zhang and Hamaker 1998; Chandrashekar and Kirleis 1988). Further, upon cooking, starch gelatinization is impeded by sorghum proteins (Xu 2008; Zhang and Hamaker 1998; Chandrashekar and others 1987; Rooney and Pflugfelder 1986). For the abovementioned reasons, the use of sorghum in food, as feed, or as an industrial energy source (i.e., starch liquefaction/ethanol production) is challenging. In order to overcome these barriers and reap the nutritional and functional benefits of sorghum starch and protein, the glutelin matrix has to be disrupted, the tight association between starch and protein bodies needs to be severed, and the structure of the protein bodies have to be broken down.

Commercial wet-milling of sorghum used to be an efficient method of obtaining a good amount of sorghum protein isolate, in addition to sorghum starch, germ and bran (Rooney and Serna-Saldivar 2000). However, this commercial operation was discontinued because of incomplete starch recovery, low oil yield, and high wax content in the grain (Yang and Seib 1995). Recently, much of the work in improving sorghum utilization has been directed towards degrading sorghum proteins via the use of reducing agents (Choi and others 2008; Ezeogu and others 2008; Ezeogu and others 2005; Elkhalifa and others 1999; Rom and others 1992) and

proteases (Zhang and Hamaker 1998; Rooney and Pflugfelder 1986). While it is true that sorghum proteins reduce starch digestibility, sorghum starch has also been shown to inhibit sorghum protein digestibility (Wong and others 2009). Thus, it is important to simultaneously disorganize sorghum starch and protein in order to maximize the digestibility of both. Technological methods for accomplishing this include popping (Correia and others 2010; Parker and others 1999) and extrusion (Mahasukhonthachat and others 2010; Zhan and others 2006; Zhan and others 2003; Hamaker and others 1994; Dahlin and Lorenz 1993a,b; Fapojuwo and others 1987; Mertz and others 1984; MacLean and others 1983). Popping, which involves dry heating of intact grain in a vessel over a steady heat source (Murty and Kumar 1995), shatters the endosperm cell walls, making starch and protein susceptible to enzymatic digestion (Parker and others 1999). Extrusion, on the other hand, uses thermomechanical energy to simultaneously reduce protein-protein and starch-protein interactions, destroy the starch crystalline structure, and gelatinize starch in sorghum endosperm so as to improve protein and starch digestibility (Mahasukhonthachat and others 2010; Dlamini and others 2009; Zhan and others 2006; Hamaker and others 1994). Fapojuwo and others (1987) found that the *in vitro* protein digestibility of whole grain sorghum flour increased from 43% to 68% after extrusion at 25% moisture content, 125 rpm and 200$^{o}$C. Zhan and others (2006) reported an increase in protein digestibility of 8% in extruded sorghum flour. Gomez and others (1988) reported a 2.25- to 2.56-fold increase in enzyme susceptible starch after extruding decorticated, nonwaxy sorghum flour at 17.5 to 44.6% moisture content.

Most studies on sorghum digestibility have focused primarily on improving sorghum starch digestibility as there is great interest in using sorghum flour for ethanol production. A key step in ethanol production from cereal flours is liquefaction. Liquefaction is typically a batch process that involves heating a 30-40% w/w aqueous slurry, adjusted to pH 6.0 to 6.5, in a jet-cooker with a thermostable α-amylase to 103-105$^{o}$C for 5 min., or to 95$^{o}$C for 1-2 h (de Mesa-Stonestreet and others 2010; Bigelis 1993). Liquefied starch is then saccharified and fermented, and ethanol is produced. Some studies on liquefaction and ethanol production also employ extrusion to enhance efficiency and throughput (de Mesa-Stonestreet and others Accepted July 2011; Vasanthan and others 2001; Govindasamy and others 1997; Grafelman and Meagher 1995). As with batch liquefaction studies, work on extrusion-liquefaction has focused only on preparing a material suitable for ethanol production, but not on utilizing the sorghum protein co-

product. While few authors prepared sorghum protein concentrates by batch liquefaction (Elkhalifa and others 2009; Duodu and others 2002a), their studies were done only on a laboratory-scale and for the purpose of protein characterization. Prior to recent work (de Mesa-Stonestreet and others Accepted July 2011), there had been no attempt to produce sorghum protein concentrates for food application by extrusion-enzyme liquefaction with a potential for large-scale production.

In a previous study using a laboratory-scale American Leistritz twin-screw extruder with a screw diameter of 18 mm (M-18, Somerville, NJ), processing conditions (in-barrel moisture content, enzyme concentration during extrusion, enzyme concentration post-extrusion) for producing sorghum protein concentrates were optimized (de Mesa-Stonestreet and others Accepted July 2011). Key findings from that work are as follows: (1) Extrusion increased the protein content (as high as 82% db) and protein digestibility (up to 66%) of sorghum protein concentrates relative to batch liquefied controls (75% db and 56%, respectively). (2) Of the three processing parameters, in-barrel moisture content had the greatest influence on specific mechanical energy (SME) and protein content. Reducing in-barrel moisture content increased SME and protein content. Although in-barrel moisture content was the primary factor affecting *in vitro* protein digestibility, its effect was confounded by enzyme concentration during extrusion, as well as protein interactions occurring during enzyme inactivation. (3) The optimum conditions for producing sorghum protein concentrates with both high protein content (75% db) and increased protein digesitibility (65%) were 32% in-barrel moisture content (SME of 593 kJ/kg) and post-extrusion addition of 2.5% flour weight basis (fwb) thermostable α-amylase.

Sorghum protein is safe for individuals suffering from celiac disease (Ciacci and others 2007) and can be used in gluten-free products to increase their protein content, and possibly improve their sensory characteristics. The primary obstacle in utilizing sorghum proteins in food is that there is presently no process that can produce highly digestible and functional sorghum protein concentrates at a large-scale. Although several small-scale laboratory procedures have been used to disrupt sorghum proteins and isolate kafirins, they had low yields and used non-food-compatible reagents (de Mesa-Stonestreet and others 2010). These laboratory experiments included the isolation of kafirins with aqueous alcohol (Bean and others 2006; Park and Bean 2003; Hamaker and others 1995; Osborne 1907), glacial acetic acid (Taylor and others 2005), and alkaline sodium borate buffer with detergent (sodium dodecyl sulfate) (Zhao and others

2008b; Ioerger and others 2007; Park and others 2006); the use of reducing agents (e.g., sodium metabisulfite, β-mercaptoethanol and glutathione) (Taylor and others 2005; Park and Bean 2003); and, sonication (Zhao and others 2008a; Ioerger and others 2007; Bean and others 2006; Park and others 2006) to increase protein extraction rates.

The abovementioned extrusion-enzyme liquefaction process described by de Mesa-Stonestreet and others (Accepted July 2011) was developed to address the abovementioned limitations in sorghum utilization. It allows for increased throughput production of sorghum protein concentrates that are more digestible and potentially more functional than protein concentrates produced by batch liquefaction. Although the laboratory-scale study (de Mesa-Stonestreet and others Accepted July 2011) established that sorghum protein concentrates produced by extrusion-liquefaction were superior to batch-liquefied protein concentrates, it remains to scale-up production of this material for commercial food applications. For this reason, the pilot-scale production of sorghum protein concentrates was performed. Using the guidance derived from previous laboratory-scale experiments, a systematic study of the critical factors affecting pilot-scale extrusion-enzyme liquefaction was conducted. The objectives of this study were to utilize a combination of processing parameters, including extrusion in-barrel moisture (MC), screw speed (RPM) and post-extrusion enzyme concentration (E), to produce sorghum protein concentrates with varying degrees of protein content and digestibility, so as to gain insight into the complex relationships between processing and end-product.

The laboratory-scale study (de Mesa-Stonestreet and others Accepted July 2011) utilized low pH (3.0) in combination with boiling for 20 min to inactivate α-amylase immediately after liquefaction. This harsh procedure led to low protein digestibility. Preliminary experiments indicated that enzyme inactivation by adding 2% fwb EDTA yielded concentrates with higher protein digestibility. Thus, the latter process was adopted for enzyme inactivation in the pilot-scale production of sorghum protein concentrate.

## 4.4  MATERIALS AND METHODS

### 4.4.1  *Materials*

The white sorghum hybrid, Fontanelle 1000, was used for this experiment. Sorghum grain was decorticated to remove 9.5% of kernel weight and then hammer-milled (1 mm screen)

in a commercial sorghum milling facility (AgVanced Enterprises, New Cambria, KS) to produce a flour with a mean particle size of 187 μm as determined by laser diffraction particle size analysis (LS™ 13 320, Beckman-Coulter, Inc., Miami, FL).  The flour had 9.51% moisture, 9.15% db protein, 1.42% db fat, 0.74% db ash, and 78.79% db carbohydrates (including 0.29% db crude fiber).

An aqueous preparation of thermostable α-amylase from *Bacillus licheniformis* with a declared activity of 240 KNU-S/g (Liquozyme SC DS, Novozymes, Franklinton, NC) was used to bring about starch liquefaction post-extrusion.  The optimum conditions for enzyme activity were pH 5.7-6.0 and 82-86$^{o}$C.  Disodium ethylenediaminetetraacetate dehydrate (EDTA; Versene Na, Dow Chemicals, USA) was used for enzyme inactivation.

### 4.4.2 *Extrusion system*

Extrusion experiments were carried out in a pilot-scale twin-screw extruder (TX-52, Wenger Manufacturing, Sabetha, KS) with a differential diameter pre-conditioning cylinder. The extruder had a screw diameter of 52 mm, length/diameter ratio of 16:1, and a circular die with an opening of 3.8 mm.  The extruder barrel had six heads.  The first five were 156 mm long and the last head was 78 mm long.  The barrel temperatures and screw profile are shown in Fig. 4.1.  Barrel temperature was 40$^{o}$C at the feed inlet and gradually increased to 110$^{o}$C at the die end.  Raw material feed rate was 80 kg/h.  Water addition was distributed between the pre-conditioning cylinder and extruder.  At 17% target MC, 2 to 3 kg/h of water was injected into the pre-conditioning cylinder and about 3 to 4 kg/h was added in the extruder.  At 32% target MC, about 10 to 11 kg/h and 15 to 16 kg/h were added in the pre-conditioning cylinder and extruder, respectively.  Extrudates were dried in a dual pass dryer (Wenger, Sabetha, KS) at 115$^{o}$C for 19 minutes and then cooled for 7 minutes to ambient temperature.

Specific mechanical energy (SME) was computed for the extrusion process as follows (de Mesa and others 2008):

$$SME(kJ/kg) = \frac{\left(\frac{\tau - \tau_0}{100}\right) \times \frac{N}{N_{rated}} \times P_{rated}}{\dot{m}} \qquad (1)$$

where, $\tau$ is the % motor torque, $\tau_0$ is the no load torque (5, 7 and 8% at 200, 300 and 400 rpm, respectively), $N$ is the screw speed (rpm), $N_{rated}$ is the rated screw speed (336 rpm), $P_{rated}$

is the rated motor power (22.37 kJ/s), and $\dot{m}$ is the total mass flow rate (kg/s). When the screw speed exceeds the rated screw speed (i.e., $N = 400\text{rpm}$), the ratio $\dfrac{N}{N_{rated}}$ equals 1.

### 4.4.3  Liquefaction

Dried sorghum extrudates were ground using a hammer mill (Fitzmill, Allen-Bradley, Elmhurst, IL) with a 50.8 micron diameter screen.  Moisture content of ground extrudates was determined and water was added to achieve an 80:20 water-to-dry matter ratio before adding the required amount of α-amylase.  The mixture was then incubated for 1 h at $82^{\circ}\text{C}$ in a water bath (Isotemp 220, Fischer Scientific, Waltham, MA) with stirring every 15 minutes.  Subsequently, the enzyme was inactivated with EDTA for at least 1 h.  The amount of EDTA added corresponded to the amount of enzyme used (i.e., 0.5, 1.5 and 2.5% fwb).

### 4.4.4  Protein Concentration

The liquefied material was centrifuged (RC2-B Superspeed, Sorvall, Newton, Connecticut) at $\sim 10{,}270g$ for 10 minutes.  The supernatant was decanted and the sediment washed three times with distilled water.  After the third wash, the sediment was freeze-dried (Freezone 6, Labconco Corp., Kansas City, MO) for 168h.  The dried sorghum protein concentrates were ground and sifted through a Tyler No. 60 sieve to achieve particle size <250 μm.  Overs were re-ground until all particles passed through the sieve.

### 4.4.5  Experimental Design

A completely randomized design (CRD) with two replicates was used to study the role of in-barrel moisture content (MC), extruder screw speed (RPM) and post-extrusion enzyme concentration (E) in obtaining sorghum protein concentrates (SPC) with high protein content (PC) and digestibility ($D_{after}$).  Protein solubility tests were also conducted to determine possible underlying differences in protein digestibility.  The processing conditions indicating the different levels of MC, RPM and E employed are shown in Table 4.1.

Sorghum protein concentrate was also prepared by batch liquefaction of sorghum flour to serve as control.  Liquefaction and protein concentration of the control and extruded treatments were carried out in the same manner.  In addition, PC and *in vitro* protein digestibility of raw

sorghum flour were determined to evaluate the overall effectiveness of the extrusion-enzyme liquefaction process.

## 4.4.6  Proximate Analysis

The proximate composition of sorghum flour and sorghum protein concentrates was determined using standard methods (AOAC, 2010; AOCS, 2009) .  This included determination of moisture ($135^{o}$C for 2h; AOAC 930.15), crude protein (based on nitrogen by combustion, 6.25X; AOAC 990.03), crude fat (petroleum ether extract method; AOAC 920.39), ash ($600^{o}$C for 2h; AOAC 942.05), crude fiber (filter bag technique utilizing $H_2SO_4$ and NaOH digestion for Ankom 200 Fiber Analyzer, Ankom Technology, Macedon, NY; AOCS Ba 6a-05), and total starch (aqueous alcohol pretreatment; amyloglucosidase/α-amylase method; AOAC 996.11). Protein, starch, fat, ash and crude fiber contents were reported as dry basis percentages (% db) from duplicate tests.

## 4.4.7  In Vitro Protein Digestibility Assay

*In vitro* protein digestibility of sorghum flour, powdered sorghum extrudates and sorghum protein concentrates were as follows.  Samples (200 mg) were dispersed in 35 mL of pepsin solution (1.5 g enzyme/L of 0.1 M potassium phosphate buffer at pH 2.0) then held at $37^{o}$C for 2 h with continuous shaking at 350 rpm using an incubator shaker (Innova 44, Pegasus Scientific Inc., Rockville, MD).  Pepsin digestion was stopped by the addition of 2 mL of 2 M sodium hydroxide solution.  The pellet collected after centrifugation at 1,110*g* for 10 min (TJ-6, Beckman Coulter, Brea, CA) was washed twice in 10 mL of 0.1 M potassium phosphate buffer at pH 2.0, and then centrifuged again using the abovementioned conditions.  After the second wash, the residue was lyophilized overnight and analyzed for protein content.  Percent *in vitro* protein digestibility, D, was computed as follows:

$$\%D = 100 \times \left[ 1 - \frac{PC_{residue} \times W_{residue}}{PC_{SPC} \times W_{SPC}} \right] \tag{2}$$

where PC is the protein content (% db) and W is the weight (mg).  Two replicates were performed for each analysis and the average digestibility was reported.

### 4.4.8  Protein Solubility

Polymeric proteins were characterized using the following multi-extraction procedure. Soluble proteins (SP) were first extracted from 100 mg of ground sample using 0.5 mL of a 12.5 mM sodium borate buffer at pH 10.0 containing 2% sodium dodecyl sulfate (SDS) (w/v), with continual vortexing for 30 min. After centrifugation at 10,000$g$ for 5 min. (Eppendorf Centrifuge 5424, Hamburg, Germany), the supernatant was removed.  The residue was sonicated (30 s at 10 W) in 0.5 mL of a 12.5 mM sodium borate buffer at pH 10.0 containing 2% SDS (w/v), and then continually vortexed for 30 min to extract the insoluble proteins (IP).  After centrifugation, the supernatant containing the IP was removed, and pellet containing residue proteins (RP) was lyophilized and analyzed for protein content.  Aliquots of the SP and IP extracts were analyzed by size exclusion high-performance liquid chromatography (SEC) using a Biosep-3000 column (Phenomenx, Torrance, CA) with 50 mM sodium phosphate buffer at pH 7.0 containing 1% SDS as mobile phase with a flow rate of 1 mL/min.  Detection of proteins was UV absorbance at 214 nm.

The SEC peak areas were summed and %RP, %SP %IP were determined as follows:

$$\%RP = 100 \times \left( \frac{PC_{RP} \times W_{RP}}{PC_{SPC} \times W_{SPC}} \right) \tag{3}$$

$$\%SP = 100 \times \%SP_{area} \times \left( 1 - \frac{\%RP}{100} \right) \tag{4}$$

$$\%IP = 100 \times \%IP_{area} \times \left( 1 - \frac{\%RP}{100} \right) \tag{5}$$

where $\%SP_{area} = 100 \times \dfrac{SP_{area}}{EP_{area}}$; $\%IP_{area} = 100 \times \dfrac{IP_{area}}{EP_{area}}$; $EP_{area}$ = total area of extracted proteins

($SP_{area}$ + $IP_{area}$); $PC_{RP}$ = protein content of the residual proteins;     $W_{RP}$ = weight of the residual proteins; $PC_{SPC}$ = protein content of the concentrate; and, $W_{SPC}$ = weight of the concentrate.

### 4.4.9  Statistical Analysis

Significant differences among the means of protein content (PC), digestibility before ($D_{before}$) after liquefaction ($D_{after}$), soluble protein content (SP), insoluble protein content (IP) and residual protein content (RP) under the different processing conditions were determined using Analysis of Variance (ANOVA).  Dunnett's test was conducted to compare each of the

processing conditions with raw sorghum flour and protein concentrate produced by batch liquefaction. Linear contrasts were constructed to compare the two moisture content levels and the three enzyme levels at constant RPM level of 300, and the two moisture content levels and the three RPM levels at constant enzyme level of 2.5% fwb. T-test was also performed to compare protein digestibility before and after liquefaction. Significance was determined when $p<0.05$ for all statistical analyses. To evaluate the linear relationship between protein digestibility after liquefaction and the percentage of residue protein, the Pearson's correlation (r) was computed. The criteria defined by (Franzblau 1958) was used to describe the degree of correlation (/r/ < 0.20, negligible; /r/ = 0.20–0.40, low; /r/ = 0.40-0.60, moderate; /r/ = 0.60-0.80, marked; /r/ > 0.80, high. The SAS 9.2 (SAS Institute, Cary, NC) software was used for all statistical analyses.

## 4.5 RESULTS AND DISCUSSION

The shearing action and thermal energy imparted during extrusion disrupts protein-protein and starch-protein interactions, disorganizes the compact starch crystalline structure, and gelatinizes starch in sorghum endosperm flour (Dlamini and others 2009; Zhan and others 2006; Hamaker and others 1994). The extent of these reactions is often described in terms of specific mechanical energy (SME). Typically, when SME values are significantly larger, the abovementioned reactions occur to a greater extent. More starch is degraded, gelatinized, solubilized at higher SME (Gropper and others 2002; Wang and others 1992). Subsequently, liquefaction by α-amylase proceeds faster (Mahasukhonthachat and others 2010). Mahasukhonthachat and others (2010) found that starch digestibility of extruded sorghum flour was 5 to 35 times faster than that of non-extruded controls. As a greater amount of starch is converted to sugars and washed away, it would be expected that purer protein concentrates (higher PC) would result. Batterman-Azcona and others (1999) related SME to the degree of disruption of zein protein bodies (corn proteins highly homologous to kafirins) in corn flour. These authors showed that SME of at least 100 kJ/kg was needed to disrupt zein protein bodies and that zein aggregated at SME >387 kJ/kg. Given that kafirins are more cross-linked than zein (Belton and others, 2006), the minimum SME required for sorghum protein body disruption is expected to be higher. Since SME has been previously used to describe the totality of reactions

occurring during extrusion, subsequent discussions on the effects of processing will utilize this parameter.

### 4.5.1 Effect of Processing Conditions on Protein Content

#### 4.5.1.1 In-barrel Moisture Content

As has been demonstrated (Mahasukhonthachat and others 2010; Agbisit and others 2007), increasing moisture content significantly reduced SME because water acts as a plasticizer. At 17 and 32% MC, average SME values were 316 and 177 kJ/kg, respectively (Fig. 4.2). PC was significantly higher at 17% MC because SME at this level was higher than SME at 32% MC. PC ranged from 73 to 80% db at 17% MC, and from 72 to 74% db at 32% MC. Processing conditions at 17% MC yielded purer protein concentrates because starch conversion was most probably more extensive at higher SME. PC of sorghum protein concentrate produced by batch liquefaction was significantly lower than the PC of all sorghum protein concentrates produced by extrusion liquefaction except at the processing condition 32% MC, 300 rpm and 2.5% fwb α-amylase.

#### 4.5.1.2 Screw speed

SME at 300 rpm was slightly lower than SME at 200 and 400 rpm at both moisture contents (Fig. 4.2). Although increasing SME is typically expected with increasing screw speed (holding other independent variables constant) (de Mesa and others 2008; Gropper and others 2002), SME reduction with increasing screw speed (150, 220 and 300 rpm) has been reported for sorghum flour extruded at 40% moisture content (Mahasukhonthachat and others 2010). Possibly, the effect of screw speed on SME within each moisture condition was so small that inherent process variability (e.g., electronic torque read-out, water injection rate, dry feed rate, pressure fluctuation) overshadowed any perceptible change in melt viscosity and SME.

The highest PC (80% db) was obtained at 17% MC and 200 rpm (Fig. 4.3A), where SME was highest (331 kJ/kg). Protein concentrates extruded at 200 rpm had significantly higher PC than those extruded at 300 and 400 rpm. In addition to having higher SME, residence time at lower screw speed is longer, which further promotes starch conversion. As previously discussed, the latter leads to faster and more efficient α-amylase liquefaction.

In spite of the relatively low SME achieved in the pilot-scale extruder, the protein contents obtained in this study were high (72 to 80% db).  In contrast, these protein levels could be achieved only at SME >450 kJ/kg in the laboratory-scale extruder (de Mesa-Stonestreet and others Accepted July 2011).  It is possible that as a result of the shear exerted in the laboratory extruder that phenolic compounds and α-amylase inhibitors that were bound in the residual bran and aleurone were released, reducing liquefaction efficiency.  For instance, condensed tannins are made more available by extrusion (Dlamini and others 2009).  Though the sample used in this study did not contain tannins, other phenolic compounds liberated by extrusion may have inhibited the action of amylases and/or bound starch granules (Taylor and Emmambux 2010), which led to reduced starch digestion (Taylor and Emmambux 2010; Bugusu 2004).

### 4.5.1.3 Enzyme Concentration

Increasing enzyme concentration at 300 rpm did not always translate into higher protein content (Fig. 4.3B).   Extrusion, given the conditions used in this study, adequately degraded starch such that only a small amount of enzyme (in this case, 0.5% fwb) appeared to sufficiently bring about extensive liquefaction.   Previous extrusion-enzyme liquefaction laboratory-scale studies also showed that raising enzyme concentration was not linearly related to protein content, although the addition of α-amylase was necessary in obtaining concentrates with protein content >26% db (de Mesa-Stonestreet and others Accepted July 2011).  While these authors used higher enzyme levels (2 to 16% fwb) than those used in the present work, both studies indicated that increasing enzyme concentration do not yield significant increases in protein content.  Thus, future studies should be to determine the level at which differentiation in protein content can be achieved.

### 4.5.2  Effect of Processing Conditions on Protein Digestibility Before Liquefaction

### 4.5.2.1 In-barrel Moisture Content

Extruding at 17% MC, but not 32% MC, significantly improved protein digestibility over that of raw sorghum flour (Fig. 4.4A).  Improvement in sorghum protein digestibility as a result of extrusion has been reported by several authors (Hamaker and others 1994; Fapojuwo and others 1987; MacLean and others 1983).  *In vitro* protein digestibility before liquefaction ($D_{before}$) at 17% MC was significantly higher (76 to 81%) than that at 32% MC (46 to 60%) (Fig. 4.4).

The relationship between protein digestibility before liquefaction and moisture content is because SME at 17% MC was higher than SME at 32%. Higher mechanical energy may have led to more extensive sorghum protein disaggregation. Similarly, Gomez and others (1988) reported that protein digestibility by pepsin was higher in samples previously extruded at 17% MC than at 32% MC. Alternatively, because there is more gelatinized starch at 32% MC as a result of having more available water, pepsin resistant complexes of retrograded starch and kafirin can form. Gelatinized starch retrogrades and forms resistant starch upon cooling, which complexes with kafirins and makes them less susceptible to enzyme attack (Duodu and others 2003). (Mahasukhonthachat and others 2010) found that degree of gelatinization was higher in conditions wherein sorghum flour was extruded at 30% MC than at 20% MC because there was more water available for starch gelatinization to take place.

### 4.5.2.2 Screw Speed

Improvement in sorghum protein digestibility as a result of extrusion has been reported by several authors (Hamaker and others 1994; Fapojuwo and others 1987; MacLean and others 1983). Results of this study indicated similarly. Extruding with 17% MC at all screw speeds significantly improved protein digestibility over that of raw sorghum flour (Fig. 4.4A). On the other hand, protein digestibility was not improved at 32% MC, and it was even significantly lower than the digestibility of raw sorghum flour when screw speed was 200 rpm. Raising screw speed did not result in a consistent increase in protein digestibility of the extrudates because only small differences in SME were also produced. Possibly, wider differences between set screw speeds would have resulted in distinguishable differences in SME and, consequently, in digestibility of the extrudates.

### 4.5.3   Effect of Processing Conditions on Protein Digestibility After Liquefaction

The protein digestibility of raw sorghum flour (58%) was not significantly different from the digestibility of the batch liquefied concentrate (57%). Extrusion liquefaction proved to be superior to batch liquefaction because *in vitro* protein digestibility values of the concentrates obtained by the former (63 to 74%) were significantly higher than those from batch liquefaction (57%) (Fig. 4.4B). In fact, the protein digestibility of raw sorghum flour (58%) was higher than that of the batch liquefied concentrate. The protein digestibilities obtained by batch and

extrusion liquefied protein concentrates were similar to those reported by Mertz and others (1984) for decorticated and decorticated/extruded sorghum flour that was cooked in excess water (200 mg ground sample in 2 mL water) for 20 minutes (57% and 79%, respectively). Hamaker and others (1994) reported similar values (61% and 79%, respectively). The disruptive effects of extrusion on sorghum protein and starch possibly facilitated pepsin accessibility, thus improved protein digestibility.

### 4.5.3.1 In-barrel Moisture Content

In general, *in vitro* protein digestibility after liquefaction ($D_{after}$) at 17% MC was lower than digestibility before liquefaction ($D_{before}$), but the converse was true at 32% MC ($p<0.05$) (Fig. 4.4C). At 17% MC, $D_{before}$ was high, indicating that proteins were disaggregated and consequently, that several reactive groups were exposed. We hypothesize that these reactive sites combined to form larger multi-peptide aggregates during liquefaction, thereby reducing protein digestibility. Results of laboratory-scale extrusion liquefaction experiments also suggested reformation of protein aggregates in the post-extrusion liquefaction and enzyme inactivation stages as the cause of reduced protein digestibility (de Mesa-Stonestreet and others Accepted July 2011). Sorghum proteins are known to form enzymatically resistant protein multimers during cooking (Duodu and others 2003), and the 1 hour liquefaction step probably gave sorghum proteins ample time to re-aggregate. Re-aggregation can occur even more quickly and more extensively in extrusion-degraded proteins because of the exposure of buried hydrophobic amino acid residues and sulfhydryl groups that can form hydrophobic associations and disulfide linkages, respectively. Fapojuwo and others (1987) also found that porridges prepared by boiling ground, extruded whole grain flour for 5 minutes at a solid-to-water ratio of 1:3 reduced protein digestibility by 21 to 25%. On the other hand, $D_{after}$ (65 to 74%) was higher than $D_{before}$ (average of 56%) at 32% MC most probably because there was more starch gelatinized, liquefied and washed out at 32% MC than at 17% MC, which made sorghum proteins more accessible to pepsin digestion. At higher moisture, more starch is gelatinized because there is more available water (Mahasukhonthachat and others 2010).

### 4.5.3.2 Screw speed

$D_{after}$ at 300 rpm was significantly lower than that at 200 and 400 rpm, while $D_{after}$ of the latter two screw speeds were not significantly different (Fig. 4.4B). This result is possibly

reflective of the fact that SME was lowest at 300 rpm. As mentioned previously, more starch and protein tend to be degraded at higher SME (Dlamini and others 2009; Zhan and others 2006; Hamaker and others 1994). Greater starch degradation leads to more extensive liquefaction and exposure of proteins. Also, degraded sorghum proteins are more easily digested by pepsin than are larger protein aggregates.

### 4.5.3.3 Enzyme Concentration

Increasing enzyme concentration was hypothesized to lead to more starch gelatinization and, consequently, higher $D_{after}$. The results, however, revealed otherwise (Fig. 4.4C). $D_{after}$ was significantly higher at lower enzyme concentrations. Rooney and Pflugfelder (1986) mentioned that gelatinized starches can form complexes with proteins that reduce protein digestibility. The reduction in protein digestibility after liquefaction seen here could be due to the formation of indigestible protein-soluble starch complexes. Sorghum proteins disrupted by extrusion may have more hydrophilic residues that could enable it to bind with starch. More enzyme produced more soluble starch available to bind with proteins, eventually resulting in lower protein digestibility after liquefaction. Because the enzyme concentration used in this study is much higher than the concentration used by other workers (Zhao and others 2008b), liquefaction probably occurred at a much faster rate and was essentially complete in a few minutes. This situation, which left even more time for soluble starch to bind with proteins, only worsened at higher enzyme concentration.

### 4.5.4  Protein Solubility

An attempt was made to explain differences in protein digestibility using the protein solubility assay that classifies sorghum proteins into soluble proteins (SP), insoluble proteins (IP) and residue proteins (RP) based on the extraction procedure by Ioerger and others (2007). Proteins soluble in SDS without sonication (SP) correspond to the albumin, globulin and kafirin-1 fraction of the Landry-Moureaux (L-M) fractionation procedure (Hamaker and others 1986) and are composed primarily of monomeric kafirins and lower molecule weight polymeric protein complexes; proteins extractable in SDS only after sonication (IP) correspond primarily to the L-M kafirin-2 fraction (larger molecular weight polymeric kafirin complexes) and contain some glutelin; and, the remaining residue protein fraction (RP) is poorly characterized and is thought to be composed of some kafirins, the L-M glutelin-like, true glutelin and unextractable proteins

(non-kafirins). The RP fraction is thought to be highly cross-linked/aggregated and nonextractable by SDS and sonication, thus is less digestible than the SP and IP fractions. A higher RP fraction is also expected from treatments producing low protein digestibility because the reduction in sorghum protein digestibility is brought about by protein polymerization (Ezeogu and others 2008; Nunes and others 2004; Duodu and others 2002a; Rom and others 1992; Hamaker and others 1986). For this reason, the RP fraction was chosen as the parameter to correlate with protein digestibility.

The correlation between protein digestibility and RP fraction at pilot-scale showed that protein digestibility after liquefaction was only moderately correlated to the RP fractions (r=0.49). The RP fraction of both batch- (78% db) and extrusion-liquefied (82 to 91% db) treatments were significantly higher than the RP fraction of raw sorghum flour (32% db). Whereas one would expect raw sorghum flour to have the highest protein digestibility because of its low RP content, its digestibility (58%) is lower than the extrusion-liquefied sorghum protein concentrates (63 to 74%) and comparable to the batch-liquefied concentrate (57%). Additionally, even though the batch-liquefied concentrate had a significantly lower RP content than the extrusion-liquefied concentrate, its protein digestibility is lower than those extrusion-liquefied. Furthermore, varying MC, RPM and enzyme concentration did not lead to a consistent trend in changes of the protein fraction percentages. Similarly, protein solubility did not reveal differences in protein digestibility nor effects of processing treatments (MC and enzyme concentration) in prior laboratory-scale extrusion-enzyme liquefaction work (de Mesa-Stonestreet and others Accepted July 2011). In the laboratory-scale study, the authors suggested that possible differences in digestibility of the RP fraction was the reason for the poor relationship between protein solubility and protein digestibility and between protein solubility and processing conditions. The results of the present work extend those conclusions to pilot-scale processing.

### 4.5.5 *Sorghum Protein Concentrates Produced at Laboratory- and Pilot-scale*

Pilot-scale extrusion consistently produced sorghum concentrates with higher protein contents and *in vitro* protein digestibilities than did laboratory-scale extrusion. Pilot-produced concentrates had protein content ranging from 72 to 80% db, and protein digestibility (after liquefaction) of 63 to 74%. In contrast, laboratory-produced concentrates had protein content

ranging from 11 to 82% db, and protein digestibility of 38 to 66%. Several differences between the two processes can be pointed out as sources of variation. First, the laboratory process had treatments wherein α-amylase was added during and after extrusion. On the other hand, all enzyme liquefaction was performed post-extrusion in the pilot-study. The introduction of α-amylase in the extruder reduced SME significantly, which led to the large SME range observed.

Second, the laboratory-extruder imparted more mechanical than thermal energy while the pilot-extruder used more thermal than mechanical energy in processing sorghum flour components. The type of energy used has implications on the conversion of starch and protein. A higher ratio of mechanical to thermal energy would induce greater starch and protein fragmentation, while a lower ratio would instead lead to more starch gelatinization and less protein disruption.

Third, the raw sorghum flour used in the laboratory study was finer (mean particle size = 119 μm) than the sorghum flour used in the pilot study (187 μm). Particle size of the raw material significantly affects extrusion and liquefaction of sorghum flour. Sorghum flour with a finer grind will have higher SME than a coarser grind (Al-Rabadi and others 2011). The high SME of the laboratory experiments can be in part due to the small particle size. Also, because fine flour would have more broken endosperm cells than coarse flour, the material used for laboratory experiments had a larger percentage of free phenolic compounds and α-amylase inhibitors than the flour used for the pilot study. As elaborated in the previous section, the phenolic compounds and α-amylase inhibitors could have contributed towards reducing liquefaction efficiency and facilitating sorghum protein aggregation. Batch liquefied concentrate derived from the fine flour had lower protein digestibility (50%) than the coarse flour (57%) also for this reason. Protein digestibility of the raw fine sorghum flour was higher than protein digestibility of the coarse sorghum flour mainly because the fine flour had more broken endosperm and fragmented sorghum proteins that were more easily accessible to pepsin. Enzyme diffusion is faster with decreasing sorghum flour particle size (Al-Rabadi and others 2009).

Fourth, the inactivation step in laboratory and pilot experiments differed. In the laboratory experiments, inactivation was done by reducing the pH of the mixture to 3.0 and boiling for 20 minutes. In pilot experiments, inactivation was done by adding 2% fwb EDTA. Preliminary experiments indicated that the latter method of inactivation produced a more

digestible protein concentrate. However, in spite of the improvement in protein digestibility observed with this inactivation method, aggregation still occurred. This was apparent when comparing protein digestibility before and after liquefaction of the protein concentrate extruded at 17% in-barrel moisture content. The reduction in protein digestibility after liquefaction was not averted by elimination of the boiling inactivation step. Liquefaction in excess water and high temperature ($82^{o}$C) promoted protein aggregation in a similar fashion to wet-cooking sorghum. This was also previously reported during batch liquefaction of sorghum for ethanol production (Zhao and others 2008b). However, inactivating α-amylase by adding EDTA instead of boiling may have reduced the extent of protein aggregation which may have occurred during boiling.

In both laboratory and pilot studies, extrusion was a critical step in obtaining sorghum protein concentrates with high purity and protein digestibility. As previously elaborated, extrusion brings about the simultaneous thermomechanical breakdown of starch and protein, which cannot be accomplished by a low-shear batch liquefaction process alone. Extrusion-enzyme liquefaction is an efficient, high throughput process because the thermomechanical pre-treatment of sorghum flour hastens starch liquefaction and removal. Additionally, extrusion disaggregates sorghum proteins, resulting in improved digestibility, and potentially better functionality. The success of the pilot-scale extrusion liquefaction study emphasizes the potential of producing sorghum protein concentrates at a commercial level.

## 4.6  CONCLUSIONS

Sorghum protein concentrates made by extrusion-enzyme liquefaction had higher protein contents (72 to 80% db) and *in vitro* protein digestibilities (62 to 74%) than did sorghum protein concentrates obtained by batch liquefaction alone (70% db and 57%, respectively). Raising in-barrel moisture content reduced specific mechanical energy, which resulted in significantly lower protein content and protein digestibility after liquefaction. Increasing extruder screw speed and enzyme concentration did not result in a linear increase in protein content and digestibility. The improvement in protein content and digestibility is attributed to thermomechanical energy imparted during extrusion processing, which degraded both sorghum starch and proteins. The proposed mechanism is that extrusion of sorghum flour simultaneously broke the intimate bond between sorghum protein and starch, disrupted sorghum proteins, disorganized the compact starch crystalline structure and gelatinized starch. The totality of these reactions appeared to

have resulted in faster and more efficient downstream starch liquefaction and removal. Results of the pilot-scale study demonstrated the potential of manufacturing high quality sorghum protein concentrates at a commercial level.

Future work includes rheological characterization of the sorghum protein concentrates. Sorghum protein concentrate is a beneficial ingredient for use in gluten-free products to improve nutritional and (potentially) functional qualities.

## 4.7  ACKNOWLEDGEMENT

# 4.8 REFERENCES

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Fig. 4.1 Screw configuration used for extrusion-enzyme liquefaction of sorghum protein concentrate.
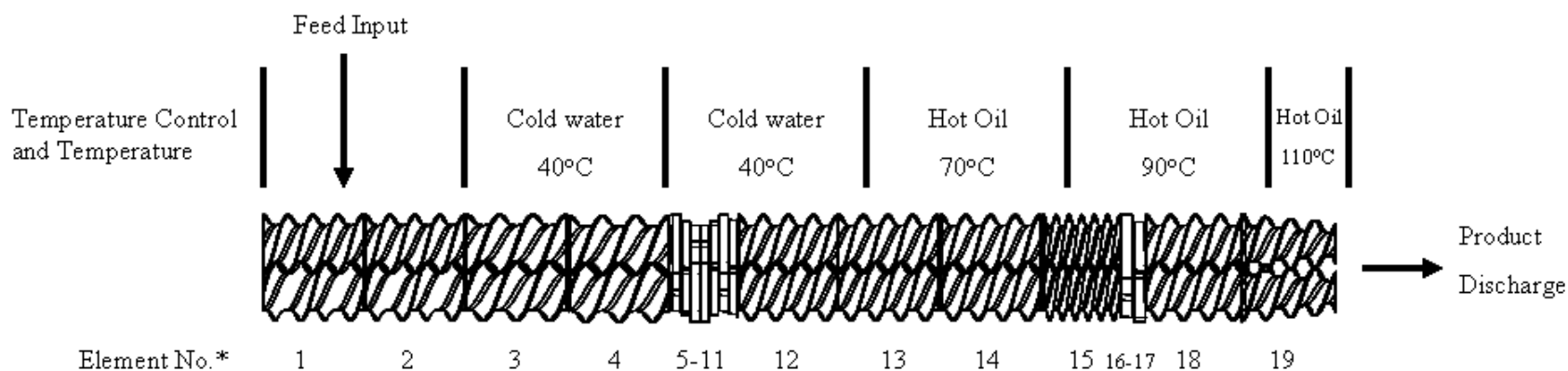
1** = SE, 0.75-2-78; 2**=SE, 0.75-2-78; 3 = SE, 1-2-78; 4 = SE, 1-2-78; 5 to 11 = KB, 7-8.70; 12 to 14 = SE, 0.75-2-78; 15 = SE, 0.50-2-52; 16 to 17 = KB, 2-8.70; 18 = SE, 0.75-2-78; 19 = Cone SE 0.75-2-78

Legend:
*All screw elements and kneading blocks are forward.
** Right shaft option: SE, 0.75-1-78
SE=screw element                         KB = kneading block
Numbers:                                 Numbers:
1st – pitch                              1st – number of disks
2nd – number of flights                 2nd – length of each disk
3rd – element length (mm)
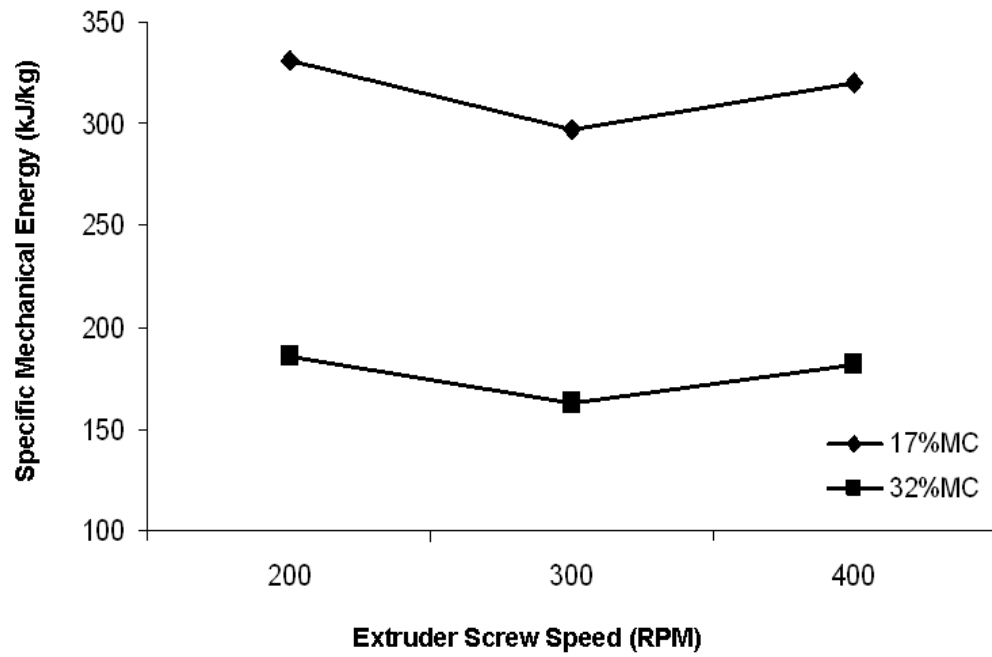
**Fig. 4.2 Effect of in-barrel moisture content and extruder screw speed
on specific mechanical energy**
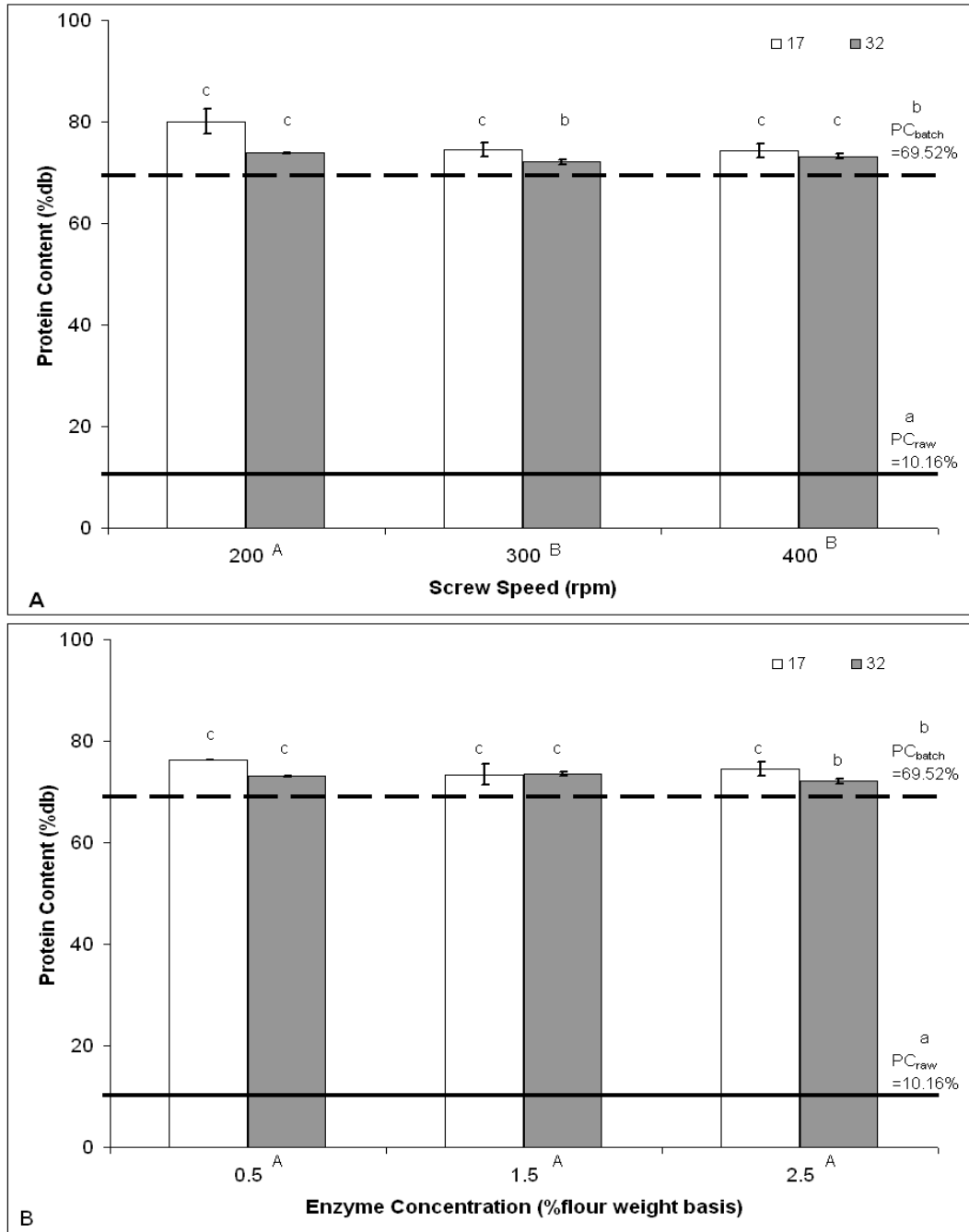
**Fig. 4.3 Effect of extruder screw speed (A, at 2.5% fwb enzyme) and α-amylase concentration (B, at 300 rpm) on protein content. Bars indicate standard deviation of two replicates. Different lower case letters indicate significant differences of the processing conditions from the batch liquefied protein concentrate and the raw sorghum flour (p<0.05). Different upper case superscript letters indicate significant difference among screw speeds (A) or enzyme concentrations (B) (p<0.05)**

A



B
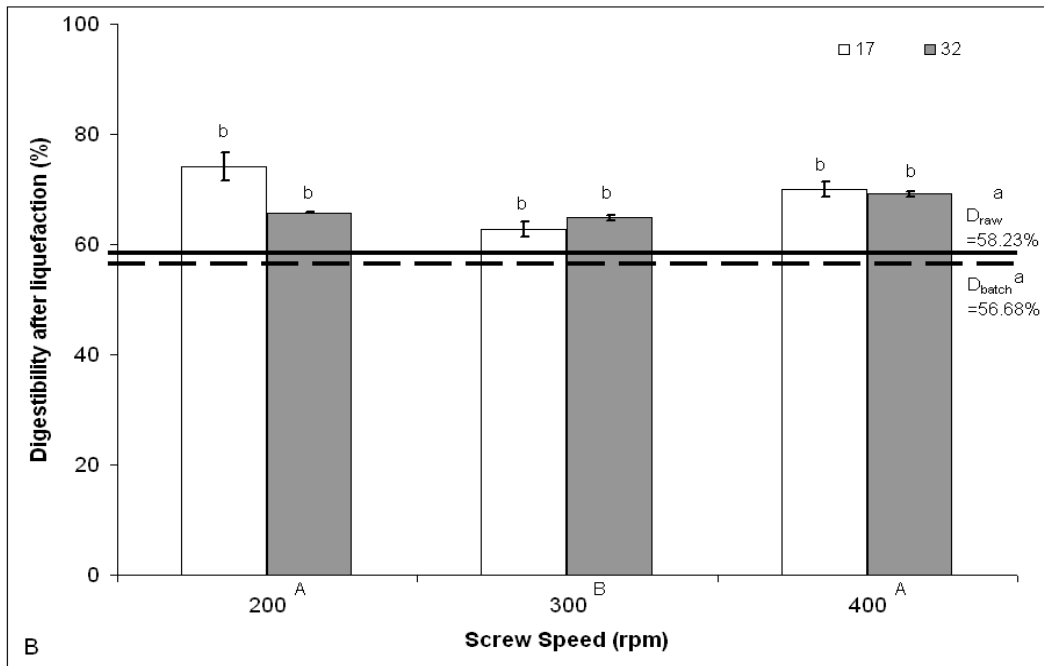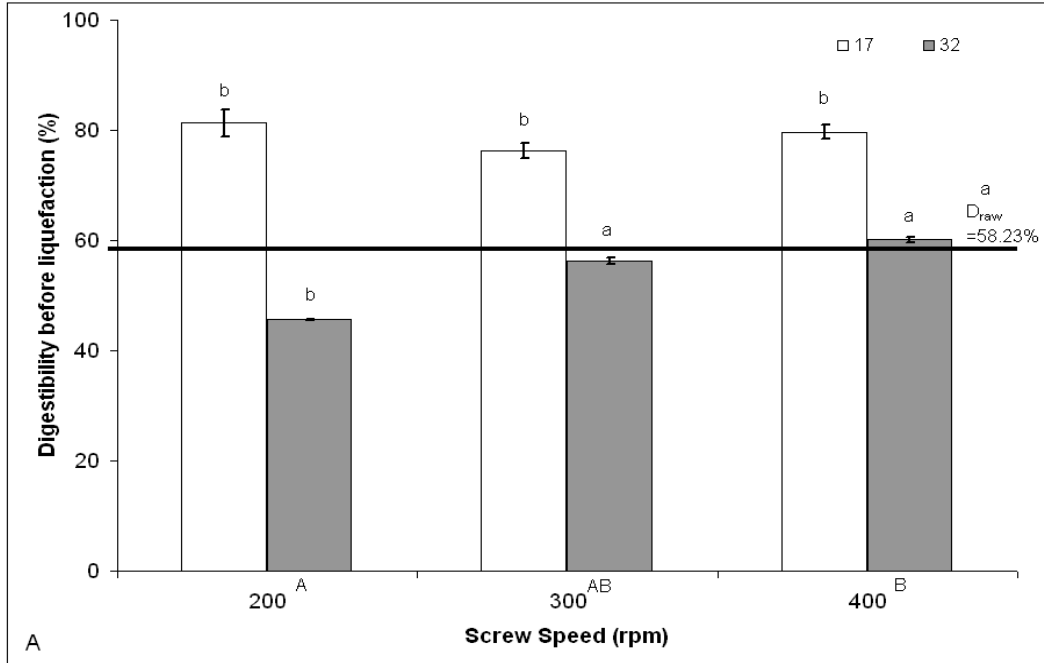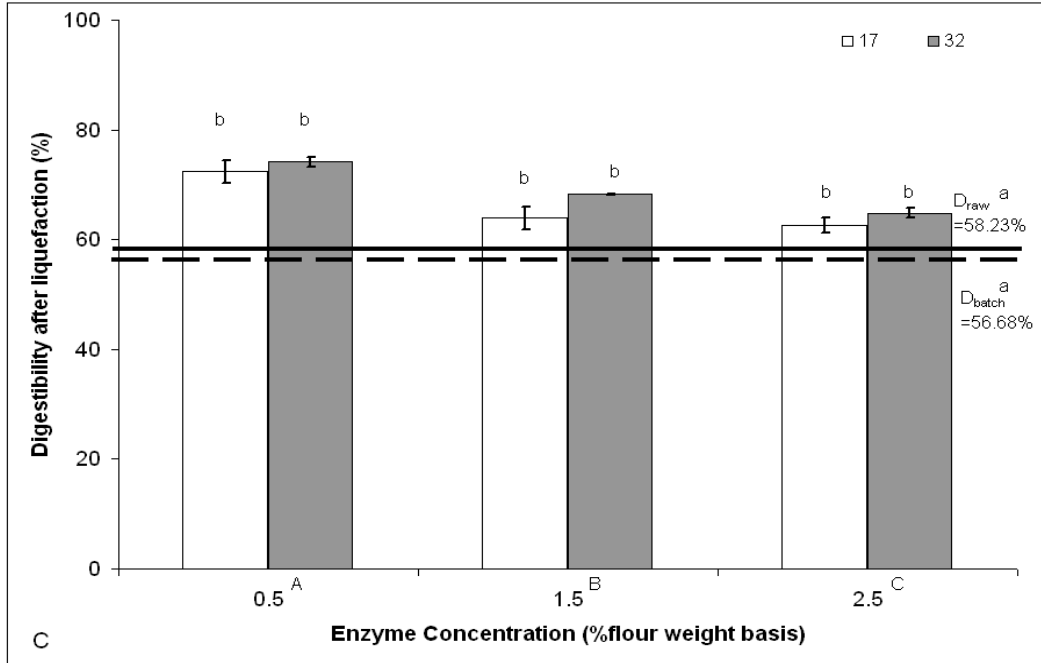
**Fig. 4.4 Effect of extruder screw speed and α-amylase concentration on *in vitro* protein digestibility before (A, no enzyme) and after liquefaction (B, at 2.5% fwb enzyme; C, at 300 rpm). Bars indicate standard deviation of two replicates. Different lower case letters indicate significant differences of the processing conditions from the batch liquefied protein concentrate and the raw sorghum flour (p<0.05). Different upper case superscript letters indicate significant difference among screw speeds (A & B) or enzyme concentrations (C) (p<0.05).**

**Table 4.1 Processing Conditions**

| In-barrel Moisture Content, % (MC) | Screw Speed (RPM) | α-amylase, % fwb (E) |
|---|---|---|
| Raw sorghum flour | N/A | untreated |
| Batch Liquefied sorghum flour | N/A | 2.5 |
| 17 | 200 | 2.5 |
| | 300 | 0.5 |
| | 300 | 1.5 |
| | 300 | 2.5 |
| | 400 | 2.5 |
| 32 | 200 | 2.5 |
| | 300 | 0.5 |
| | 300 | 1.5 |

# Chapter 5 - Rheological characterization of sorghum protein concentrates produced by extrusion-enzyme liquefaction and its application in dough and batter-based food systems

## 5.1 ABSTRACT

Dynamic oscillatory measurements (amplitude and frequency sweep) of two sorghum protein concentrates produced by extrusion-enzyme liquefaction at low (17% dry basis, db) and high (32% db) in-barrel moisture contents were evaluated.  Sorghum protein concentrate (5 and 10%, based on starch) was added to potato starch and two levels of moisture content were used (55 and 65% db) to simulate dough and batter food systems.  In the protein concentrate-potato starch dough system, the concentrate extruded at 17% in-barrel moisture consistently had higher G' and G" values than the concentrate extruded at 32% in-barrel moisture, regardless of addition level.  Also, adding more protein concentrate resulted in higher G' and G", which approached the G' and G" of pure starch dough.  When applied to sorghum tortilla dough, sorghum protein concentrate improved tortilla quality, with tortillas containing protein concentrate 17 being superior to those with concentrate 32.  Moreover, higher level (10%) of addition significantly improved the quality of the tortillas.  In the protein concentrate-potato starch batter system, potato starch exhibited significantly higher G' and G" than any of the batters with sorghum protein concentrate.  These rheological results are reflective of the detrimental effect of sorghum protein concentrate to the quality of sorghum, batter-based bread.    Possibly, the protein concentrate disrupted the starch gel which kept the structural integrity of the bread batter.

## 5.2 INTRODUCTION

Gluten-free breads using sorghum flour have poor volume compared to wheat breads. Furthermore, the addition of sorghum flour reduces loaf volume in sorghum wheat composite breads (Bugusu and others 2001).  One of the factors contributing to the inability of sorghum to produce bread with quality comparable to that of wheat is related to the encapsulation of sorghum prolamins (kafirins) in rigid protein bodies, which makes them unavailable for interaction and limits their ability to form a protein network (Bugusu and others 2001).  Zein is the corn prolamin that is highly homologous to kafirins.  When added in its free form, i.e., free from protein bodies, zein (10%) can form a viscoelastic dough with 90% corn starch and 75% water (based on zein-starch weight) (Lawton 1992).   A sorghum-wheat composite bread that was comparable to 100% wheat bread was produced with the addition of free-form zein (Bugusu and others 2002; Bugusu and others 2001) because free-form zein was available to cross-link and form thin protein fibrils.  These protein fibrils contributed to the formation of a viscoelastic

protein network responsible for good dough strength and loaf volume (Bugusu and others 2002). Similarly, in gluten-free bread made primarily from zein and corn starch, the zein protein network was responsible for the physical stability, extensibility and cohesiveness of dough (Schober and others 2008). Schober and others (2008) found that zein fibrils formed only at high viscosity, which was achieved either by reducing water or with the addition of hydroxypropyl methylcellulose (HPMC). These authors also noted that the formation of zein fibrils (or strands) alone does not guarantee satisfactory gas holding capacity and that a surface-active agent such as HPMC was necessary in order to stabilize the gas-liquid interface of gas cells. Due to its similarity to zein, it was suggested that kafirins, if freed from protein bodies, could also form viscoelastic networks that have the ability to entrap carbon dioxide released by yeast during dough fermentation (Bugusu and others 2001).

Sorghum protein concentrates were produced by extrusion-enzyme liquefaction to disrupt sorghum protein bodies, liberate kafirins and make them more available for interaction (de Mesa-Stonestreet and others Accepted July 2011). Extrusion has been shown liberate zein from protein bodies in maize (Batterman-Azcona and others 1999). By the same mechanism, kafirins were most probably also freed up during the extrusion process (de Mesa-Stonestreet and others Accepted July 2011; de Mesa-Stonestreet and others In review). Extrusion pre-treatment of sorghum flour followed by α-amylase liquefaction and protein concentration resulted in sorghum protein concentrates having higher protein content and protein digestibility than either raw sorghum flour or batch-liquefied sorghum flour. These parameters indicate that sorghum protein concentrates produced by extrusion-enzyme liquefaction are more available for interaction (and potentially more functional) than untreated sorghum proteins. Thus, the main goal of this study was to evaluate the fundamental rheological properties of a model system composed of potato starch and sorghum protein concentrate. Rheological investigations were performed at low (55% dry basis or db) and high (65% db) moisture contents to simulate dough and batter-based bread systems, respectively. Additionally, two sorghum protein concentrate levels (5 and 10%, based on starch) were used to determine concentration effects. Sorghum protein concentrates were added to sorghum-based tortilla dough and bread batter to draw a relationship between fundamental rheological properties and end use.

## 5.3  MATERIALS AND METHODS

### 5.3.1  Rheological Tests

#### 5.3.1.1 Preparation of sorghum protein concentrate

The two sorghum protein concentrates used in this study were produced in a pilot-scale extrusion-enzyme liquefaction process described by de Mesa-Stonestreet and others (Accepted July 2011).  In this process, decorticated sorghum flour was extruded and then liquefied with thermostable α-amylase.  After 1-h liquefaction, the enzyme was inactivated and soluble starch was removed by a series of centrifugation and water washing steps.  The remaining protein concentrate was freeze-dried and ground.  The resulting protein concentrates had particle size of less than 125μm.  The sorghum protein concentrates used for rheological work were extruded at 17 and 32% db in-barrel moisture content (MC), and referred to as protein concentrate 17 and 32, respectively, throughout the text.  The protein content (based on nitrogen by combustion, 6.25X; AOAC 990.03, AOAC 2010) of the concentrates were 74 and 72% db, respectively; and, *in vitro* protein digestibility values were 76 and 56%, respectively.  Moisture contents ($135^{o}$C for 2h; AOAC 930.15, AOAC 2010) were 17.3%, 6.4% and 6.2% for potato starch and sorghum protein concentrates 17 and 32, respectively.

#### 5.3.1.2 Preparation of protein-starch blends

Unmodified normal potato starch was procured from Bob's Red Mill (Milwaukie, OR). Sorghum protein concentrate and potato starch blends were prepared with 0% fwb (pure potato starch), 5% (flour weight basis, fwb) and 10% fwb sorghum protein concentrate.  The protein contents of the dry blends were 4.05% db (5% fwb of concentrate 17, also '17-5'), 7.78% db (10% fwb of concentrate 17, also '17-10'), 3.93% db (5% fwb of concentrate 32, also '32-5'), and 7.56% db (10% fwb of concentrate 32, also '32-10').  The powders were mixed twice (30 s each time) in a 5-g capacity mixograph at 85 rpm.  The mixograph bowls were scraped with a rubber spatula in-between mixing.  Two water addition levels were used to simulate dough (55% db) and batter (65% db) systems.  The required amount of distilled water (~$95^{o}$C) was added to the dry blend in the mixograph bowl.  The pastes were mixed thrice (30 s each time) using the mixograph settings previously mentioned.  The mixograph bowls were scraped with a rubber

spatula in-between mixing.  After mixing, the pastes were placed in airtight containers and allowed to rest for 5 min before measurement.

### 5.3.1.3 Dynamic oscillatory tests

The dynamic rheological properties of all samples were evaluated using a Bohlin CVO Rheometer (Malvern Instruments Ltd., Worcestershire, UK) with a 20 mm smooth parallel plate. Sample was placed between the plates, the gap adjusted to 1 mm, and the edges trimmed with a rubber spatula.  The edge of the sample was covered with a thin layer of silicone oil (RT10, Cannon Instrument Company, State College, PA) to prevent water evaporation, and the sample was allowed to rest between the plates for 3 min at 30$^{o}$C prior to testing to allow residual stresses to relax.

Amplitude sweep test was performed at strain values from $1 \times 10^{-5}$ to 0.10 at a frequency of 1 Hz in order to establish the linear viscoelastic region.  A frequency sweep test from 0.01 to 100 Hz was performed at a constant strain of 0.0005 and 0.005 for dough and batter, respectively.  Preliminary amplitude sweep test at 1 Hz indicated that the strain values used for dough and batter samples were within the linear viscoelastic region.  All measurements were conducted at 30$^{o}$C.  All rheological experiments were performed three times and their averages were reported in the study.

### 5.3.2   Tortilla study

### 5.3.2.1 Raw Materials

In making sorghum tortillas, debranned, white sorghum flour (Archer Daniels Midland Company, TX) which had 11.76% moisture content (135$^{o}$C for 2h; AOAC 930.15), 10.10% db protein (based on nitrogen by combustion, 6.25X; AOAC 990.03),  1.94% db crude fat (petroleum ether extract method; AOAC 920.39), 1.28% db fiber ($H_2SO_4$ and NaOH digestion, AOCS Ba 6a-05), 86.02% db starch (glucoamylase method; AOAC 979.10), and 0.35% db ash (600$^{o}$C for 2h; AOAC 942.05) was used.  The proximate composition of sorghum flour was determined using standard methods (AOAC, 2010; AOCS, 2009) .  Granulated sugar, vegetable shortening, glycerin, salt, xanthan gum, double acting baking powder and citric acid and monoglyceride (Dimodan PH300 K-A, Danisco, New Century, KS) were used in the proportions specified in Table 5.1.  The sorghum flour tortilla formulation developed by Fernholz (2008) was

adopted with modifications on the brand of sorghum flour, amount of water added and addition of sorghum protein concentrates. The sorghum protein concentrates were added at 5 and 10% based on sorghum flour weight.

### 5.3.2.2 Experimental design and statistical analysis

A randomized complete block design with two factors (type of sorghum protein concentrate and percentage addition) and two levels for each factor was used. The treatments are identified as 17-5, 17-10, 32-5 and 32-10 to indicate the type of sorghum protein concentrate used and the percent added (based on sorghum flour weight). Experiments were blocked by day. A control formulation not containing sorghum protein concentrate was also made on each day. Tortillas were evaluated for moisture content, specific volume, rollability, extensibility and stretchability. There were five subsamples for specific volume and two for all other parameters. Dunnett's test was performed to determine which treatments were significantly different from the control. Analysis of Variance (ANOVA) was performed to determine significant differences among treatment means. Where significant differences exist, Tukey's multiple comparison test was performed. Significant differences were determined at $p \leq 0.05$. Statistical analyses were performed using the SAS software (v.9.2, SAS Institute Inc., Cary, NC).

### 5.3.2.3 Tortilla preparation

A Hobart 3-speed table top mixer (Model N-50, Hobart, Ontario, Canada) was used to mix tortilla dough. Vegetable shortening and glycerin were mixed for 30 sec at speed 1. The sides of the bowl were scraped down with a spatula and mixing continued for 30 sec at speed 2. All dry ingredients except sorghum flour were added and mixed for 30 sec at speed 1 and then for another 30 sec at speed 2, with scraping in-between mixes. Sorghum flour and warm ($38^{o}$C) water were added and mixed for 30 sec at speed 1. The sides of the bowl were scraped down with a spatula and mixing continued for 60 sec at speed 2. The dough was divided into 25 g dough balls and kept in a lidded container until use to limit moisture loss. Dough balls were used within 30 min from forming.

Sorghum dough balls were pressed using a DoughPro (ProProcess Corporation, Paramount, CA) with a thickness setting of 1.5 mm at $113^{o}$C for 2 sec. Flattened tortilla dough was grilled immediately on a table top griddle (Model 8200, Gold Medal, Cincinnati, OH) at

190$^{\circ}$C for 30 s on each side.  Tortillas were cooled for 2 min and then stored in polyethylene bags.  Tortillas were evaluated 3 to 4 hours after cooling.

### 5.3.2.4 Tortilla evaluation

#### 5.3.2.4.1  Moisture content

Tortilla moisture content was determined using the standard two-stage procedure (50$^{\circ}$C, 12h then 130$^{\circ}$C, 1h; AACC 44-15.02 Approved November 3, 1999).

#### 5.3.2.4.2  Specific volume

Specific volume (SV) was calculated from the diameter (cm), thickness (cm) and weight (g) of each tortilla.  The diameter of each tortilla was the average of the long and short measurements.  The thickness of each tortilla was the average of two measurements.  Specific volume (cm$^3$/g) was calculated as below.  Five tortillas were evaluated for each treatment.

$$SV = \frac{\pi \times \left(\dfrac{\text{Diameter}}{2}\right)^2 \times \text{Height}}{\text{Weight}}$$

#### 5.3.2.4.3  Rollability

Rollability was evaluated by wrapping a tortilla around a dowel.  Dowels with 2.5 cm, 1.5 cm and 1.0 cm diameters were used.  The cracking and breakage of the tortilla was rated using a scale from 1 to 5, where 5 = no cracking; 4 = signs of cracking, but no breaking; 3 = cracking and breaking beginning on the surface; 2 = cracking and breaking imminent on both sides; 1 = unrollable, breaks easily.  Two tortillas were evaluated for each treatment.

#### 5.3.2.4.4  Extensibility

A tortilla strip measuring 3.5 cm x 3.7 cm was cut out of the center of the tortilla with a carving knife.  Extensibility of a tortilla strip was evaluated using a texture analyzer (TA-XT2, Texture Technologies Corp., Scarsdale, NY) with tensile grip attachments (TA-96 double clamp set).  Force in tension was measured.  Pre-test and test speeds were 1.00 mm/s, while post-test speed was 5.00 mm/s.   Distance was 25.00 mm and trigger force was 5 g.  Recorded Peak force (g) and distance (mm) corresponded to the rupture force and distance to tear of the tortilla.  Two tortillas were evaluated for each treatment.

### 5.3.2.4.5  Stretchability

Stretchability of tortilla was evaluated using a texture analyzer (TA-XT2, Texture Technologies Corp., Scarsdale, NY) with a TA-108 tortilla/film fixture and TA-108a (18 mm diameter spherical probe) and following the American Institute of Baking (AIB, Manhattan, KS) standard procedure.  Force in compression was measured.  Pre-test, test and post-test speeds were 6.0 mm/s, 1.7 mm/s and 10.0 mm/s, respectively.  Distance was set at 30.0 mm and trigger force was 20g.  Peak force (g) and distance (mm) were reported.  Two tortillas were evaluated for each treatment.

## 5.3.3  Bread study

### 5.3.3.1 Materials

Decorticated sorghum flour with a mean particle size of 187 μm as determined by laser diffraction particle size analysis (LS™ 13 320, Beckman-Coulter, Inc., Miami, FL)  was used for breadmaking.  The flour had 9.51% moisture, 9.15% db protein, 1.42% db fat, 0.74% db ash, and 78.79% db carbohydrates (including 0.29% db crude fiber).  The proximate composition of sorghum flour was determined using standard methods (AOAC, 2010; AOCS, 2009) as indicated above (5.3.2.1).  Unmodified native potato starch was obtained from Bob's Red Mill (Milwaukee, OR) and hydoxypropylmethylcellulose or HPMC (Methocel K4M) was sourced from Dow Chemical Co. (Midland, MI).  Table salt, granulated sugar and active dry yeast were procured locally.

### 5.3.3.2 Experimental design and statistical analysis

Breadmaking experiments followed a randomized complete block design with two factors (type of sorghum protein concentrate and percentage addition) and two levels for each factor were used.  The treatments were identified as 17-5, 17-10, 32-5 and 32-10 to indicate the type of sorghum protein concentrate used and the percent added (based on potato starch weight).  Experiments were blocked by day.  A control formulation not containing sorghum protein concentrate was also made.  Sorghum breads containing sorghum protein concentrates collapsed and so the experiments were terminated and the breads were no longer evaluated.

### 5.3.3.3 Standardization of batter water addition

Batter consistency was standardized with a texture analyzer (TA-XT2, Texture Technologies, Scarsdale, NY) using a forward extrusion cell (TA-93) with a 10-mm nozzle washer set on top of a platform (TA-90) following the procedure by Schober and others (2005) with slight modifications. Modifications made included reducing the amount of batter used and increasing the number of water levels used. For extrusion tests, 50 g of batter was prepared using the control formulation in Table 5.2 based on the work of Frederick (2009), with the exception of yeast. The control batter (no protein concentrate) was extruded at 100, 105, 110, 115 and 120% (flour weight basis) water levels. In this case, the weight of sorghum flour, potato starch and sorghum protein concentrate were added and interpreted as flour weight basis (fwb). The batter was mixed with a hand mixer to ensure homogeneity and then immediately loaded into the extrusion cell. Air pockets were removed with a metal spatula and the batter was pre-compressed. Pre-test and test speeds were 1 mm/sec, and post-test speed was 10 mm/sec. Trigger force was 50 g. Extrusion force was measured over a distance of 20 mm and the average force after reaching a plateau (8 to 18 mm) was used to indicate batter firmness. The measurement was repeated three times with the same batter and the average of the three readings was considered 1 replicate. Two replicates were performed.

Control sorghum breads were made using the formulation in Table 5.2 with 100, 105, 110, 115 and 120% fwb following a completely randomized design. Two replicates were performed. Specific volume (mL/g) and loaf height (mm) were evaluated. Statistical analysis indicated that varying moisture levels did not result in significant differences in specific volume and loaf height. A moisture level of 105% fwb was chosen because this was also the selected water level in prior work on gluten-free breads (Schober and others 2007).

Treatments containing protein concentrate were extruded at 105, 110 and 120% fwb water. Using the extrusion force of the control batter with 105% fwb water as the selection criterion, the amount of water added to treatments containing protein concentrate was interpolated.

### 5.3.3.4 Breadmaking

Breadmaking experiments used the formulation described by Frederick (2009) as control. Protein concentrate was added based on the weight of potato starch. As previously described, water was added to treatments with protein concentrate was based on the amount needed to reach the same consistency as the control. The formulations are shown in Table 5.2.

In preparing the batter, sugar was first dissolved in the specified amount of water for each treatment. This sugar solution (30°C) was used to re-hydrate dried yeast for 5 min. The remaining dry ingredients were mixed separately for 5 min using a Hobart mixer (Model N-50, Hobart, Ontario, Canada) with a flat beater attachment at speed 1 of 3. The water-yeast mixture was added to the dry ingredients and mixed for 30 s at speed 1, and then scraped. Mixing was continued for 90 s at speed 2. Batter amounting to 250g was weighed into greased baking tins (width = 9 cm, length = 15 cm, height = 5.5 cm) and proofed at 32°C and 85% relative humidity in a proofing cabinet (National Manufacturing Co., Lincoln, NE). Each batter was proofed to height, i.e., 1 cm below the edge of the tin. Proof time was about 30 min. From the proofer, batters were baked for 30 min in a reel-type baking oven (National Manufacturing Co., Lincoln, NE), which was pre-heated to 232°C. After baking, the loaves were depanned and cooled for 1.5 h on cooling racks at ambient temperature.

### 5.3.3.5 Bread evaluation

After cooling, the loaves were weighed and loaf volume was measured by standard AACC method 10-05.01 (rapeseed displacement; Approved October 17, 2001). Loaf specific volume (mL/g) was calculated as:

$$SV = \frac{\text{loaf volume}}{\text{loaf weight}}$$

Loaf height of the middle slice was determined by high definition imaging using the C-Cell (Calibre Control International Ltd., Appleton, Warrington, UK). Only the control loaves were evaluated for volume and height during the moisture standardization tests because experiments evaluating for the effect of protein concentrates were terminated.

## 5.4 RESULTS AND DISCUSSION

### 5.4.1 Rheological tests

### 5.4.1.1 Dynamic rheological properties of dough

Frequency sweep results for dough samples are presented in Fig. 5.1. The plots show that magnitudes of G' and G" increased with increasing frequency, and G' was greater than G". Both moduli were frequency dependent. Sorghum protein concentrate extruded at 17% MC

consistently showed higher G' than the concentrate extruded at 32% MC, regardless of concentration. This indicates that the former has a more solid-like structure than the latter. The effects of protein content and digestibility of the concentrates on dynamic rheological properties are discussed in detail in Section 5.4.1.2. For both concentrates, higher protein concentrate ratios led to higher G' and G". Protein competes with starch for available water, and the presence of which reduced the amount of available water, making the paste more solid-like. Additionally, pastes with 5% protein concentrate were more frequency dependent than pastes with 10% protein. This indicates that raising the amount of protein improves the elasticity of the paste. The increase in elasticity at higher protein concentration is probably reflective of a greater number of protein-protein and/or starch-protein interactions taking place.

The G' and G" of potato starch closely matched those of both concentrates at 10% addition. Based on these results, it is expected that this level of addition would improve the quality of starch-based, gluten-free doughs, such as tortilla dough. In sorghum tortilla, the use of protein concentrate 17 instead of protein concentrate 32 can be expected to result in a better product because the G' and G" values of the former were higher than those of the latter. A material with higher G' could make tortillas withstand greater extension and shearing forces, while a higher G" can make tortillas more pliable and rollable.

### 5.4.1.2 Dynamic rheological properties of batter

Frequency sweep results for batter samples are presented in Fig. 5.2. As with dough frequency sweep curves, protein concentrate extruded at 17% MC exhibited higher G' and G" than that extruded at 32% MC, regardless of concentration. Also, both moduli are higher at higher protein concentrate levels. In contrast to dough frequency sweep curves, potato starch had higher G' and exhibited less frequency dependency than the starch-protein concentrate mixtures, possibly indicating greater stability in applications with high water content. A material showing more stability over a wide frequency range is desirable because it indicates fewer molecular rearrangements (Onyango and others 2011). Starch-protein concentrates, especially the concentrate extruded at 32% MC, appeared to show instability sooner than potato starch samples. That is, plots of starch-protein concentrates showed a sudden, steep, upward shift in G' and G" at frequencies starting at 3.5 Hz, whereas this occurred in potato starch batter beginning at 43 Hz. The presence of the protein concentrate most probably disrupted the starch gel and weakened its structure. This effect was greater in protein concentrate 32 possibly because of its

protein content and the nature of its proteins.  Sorghum protein concentrate 17 had higher protein content and was more digestible.  Having higher protein content, sorghum protein concentrate 17 bound more water and increased batter viscosity.  Being more digestible, the concentrate extruded at 17% MC probably had more sites available to interact with starch, and/or were more easily dispersed in the starch matrix without compromising the integrity of the latter.  This concentrate appeared to form a stable starch-protein network.  In contrast, being a less digestible protein concentrate, protein concentrate 32 had longer protein peptides and less available reactive sites that can interact with starch.  And, possibly because of the bulky structure of its peptides, protein concentrate 32 would be less likely to disperse evenly in the starch matrix without weakening it.  Likewise, when using 2.4% (w/w db) whey protein in making gluten-free bread containing wheat starch (92.6%, w/w db), locust bean gum (0.4%, w/w db), salt (2.0% w/w db), glucose (0.9% w/w db) and yeast (1.7%, w/w db), adding whey protein particles with 100nm to 100µm length instead of whey protein aggregates produced  gluten-free bread with larger specific volume and loaf height, and more even crumb (van Riemsdijk and others 2011).  This study demonstrated the relevance of protein particle size in creating an even starch-protein matrix, which is important in gluten-free bread development.

The supplementation of gluten-free breads with sorghum protein concentrate also requires sufficiently high viscosity in order to obtain an acceptable product.  As seen in the rheological characterization of the starch-sorghum protein concentrate batters, it was not possible to obtain stability with starch and protein concentrate alone.  Additives like hydroxypropyl methylcellulose, which has been used in gluten-free breads (Schober and others 2008; Schober and others 2007; Hart and others 1970), can probably increase the viscosity of gluten-free bread batters supplemented with sorghum protein concentrate.

Although extrusion-enzyme liquefied sorghum protein concentrates are rich sources of free-form kafirins, they are still unable form a viscoelastic protein network similar to that formed by zein.  This is possibly because of the presence of other interfering materials in the protein concentrate such as fiber, fat, residual starch, sugars and ash.  Zein is about 89% db protein.  Hence, in order to achieve functional properties similar to those of zein, sorghum protein concentrates need to be further purified.  It is suggested that future work focus on isolating sorghum proteins from sorghum protein concentrates.

### 5.4.2 Tortilla study

Sorghum protein concentrates were added to a sorghum-based tortilla recipe to evaluate the ability of the concentrates to improve the quality of dough food systems. Protein supplementation of tortillas is a desirable method of improving its nutritional quality (Racotta and others 1979). Statistical analyses showed that the interaction between the type of sorghum protein concentrate and the percentage of addition was not a significant factor affecting any of the quality parameters. This means that these two factors acted independently. Table 5.3 shows which physico-chemical properties were significantly affected by protein concentrate type and percentage addition. All parameters except for specific volume and rollability at 2.5 cm and 1.5 cm were significantly influenced by protein type and percentage addition. Discussion of each parameter follows.

#### 5.4.2.1 Moisture content

The control had the lowest moisture content of all treatments. Moisture contents of tortillas containing sorghum protein concentrates were higher, with the exception of treatment 32-5 (Table 5.4). Protein type and addition level significantly affected tortilla moisture content (Table 5.3). Tortillas containing protein concentrate 17 had significantly higher moisture content than tortillas with concentrate 32. Protein concentrate 17 absorbed more water than concentrate 32 probably because it had more hydrophilic groups exposed as a result of having higher *in vitro* protein digestibility. Higher levels of protein concentrate also produced tortillas with higher moisture content. Tortillas with protein concentrate 17 at 10% addition had the highest moisture content.

#### 5.4.2.2 Specific volume

The treatments did not result in significant differences in specific volume (Table 5.3 and Table 5.4). Thus, tortilla production was reproducible and tortillas from all treatments were reasonably uniform. This also implies that differences in other quality parameters were not affected by variations in the weight and dimensions of individual tortillas.

#### 5.4.2.3 Rollability

Rollability using 2.5 cm and 1.5 cm diameter dowels did not differentiate treatments (Table 5.3 and Table 5.4). Using a 1.0 cm diameter dowel, however, showed that tortillas

containing protein concentrate 17 were more rollable than tortillas containing protein concentrate 32. Adding 10% protein concentrate also improved rollability. These results appear to be related to moisture content. That is, more rollable tortillas had higher moisture content, possibly because tortillas with higher moisture content were softer and more pliable. The control had the lowest moisture content and also the lowest rollability score.

### 5.4.2.4 Extensibility

Tortillas containing protein concentrate 17 had lower rupture force than those containing protein concentrate 32. Adding 10% protein concentrate also lowered rupture force. Lower rupture force appears to be related to higher moisture content. Tortillas with higher moisture content are softer and require less force to break. Fernholz (2008) also reported that sorghum tortillas with higher moisture content are more pliable. The control tortilla had the lowest moisture content and the highest rupture force. Tortilla containing protein concentrate 17 appeared more extensible than tortilla with protein concentrate 32 because it was able to travel a longer distance before tearing and it required the least amount of force to tear. Hard and firm tortillas have a higher rupture force than soft tortillas (Suhendro and others 1999). On the other hand, adding 10% protein concentrate did not result in longer distance to tear even though it had a lower rupture force. This implies that although tortillas with higher protein content were softer, they did not extend as much as those with only 5% protein concentrate.

### 5.4.2.5 Stretchability

Results indicated that tortillas with protein concentrate 17 were more rubbery than those with protein concentrate 32 because the former required significantly larger force to stretch, and did so for a longer distance. The same can be said for tortillas with 5% protein concentrate, i.e., these tortillas required larger force to stretch a longer distance.

Data indicated that protein concentrate 17 yielded tortillas that were more extensible and stretchable. Also, while higher protein levels resulted in more rollable tortillas, these were not very extensible nor stretchable.

### 5.4.2.6 Relationship between rheological properties and tortilla quality

Tortilla rollability scores indicated that using sorghum protein concentrate 17 and adding 10% protein concentrate made sorghum dough stronger. Results of the rheological study of

dough (55% water) containing sorghum protein concentrate and potato starch (Fig. 5.1) support these observations. The frequency sweep showed that the elastic and viscous moduli of protein concentrate 17 were higher than those of protein concentrate 32, in general. Both elastic and viscous moduli were also higher at 10% protein concentrate.

### 5.4.3 Bread study

#### 5.4.3.1 Standardization of batter water addition

Baking tests showed no significant differences in specific volume and loaf height of the control sorghum breads containing 100, 105, 110, 115 and 120% water. Since previous work by Schober and others (2007) used 105% water, this level was selected for standardizing batter consistency. Results of the extrusion cell tests indicated that the control batter formulation with 105% water level had an average extrusion force of 12 Newtons. In order to obtain the same force, the amount of water added to the treatments were increased to 108 and 110% when 5 and 10% protein concentrate were added, respectively.

#### 5.4.3.2 Breadmaking

Sorghum bread using the control formulation with 105% water had an average specific volume of 2.65 mL/g and a loaf height of 57 mm. This value is comparable to the specific volume of sorghum bread reported by Schober and others (2007). The addition of sorghum protein concentrate resulted in a collapse of the bread. For this reason, the experiment was discontinued and no further evaluation was conducted.

#### 5.4.3.3 Relationship between rheological properties and bread quality

The inability of sorghum protein concentrates to improve the quality of sorghum batter-based bread is reflected in its rheological properties. The addition of sorghum protein concentrate resulted in significantly lower elastic and viscous moduli than those of pure potato starch, regardless of protein concentrate type and concentration (Fig. 5.2). The collapse of sorghum bread containing protein concentrates is probably due to the inability of sorghum proteins to form a viscoelastic network as elaborated above (5.4.1.2) and/or due to the suppression of HPMC functionality. The addition of proteins, such as soy protein isolate and egg white protein, to HPMC-treated rice cassava bread was found to reduce dough stability by suppressing HPMC functionality (Crockett and others 2011a). Crockett and others (2011) found

that soy and egg proteins altered water distribution within rice cassava bread, weakened HPMC interactions with starch and reduced foam stability. The hydrophilic hydroxypropyl side groups of HPMC interact with starch and water by hydrogen bonding, while the presence of hydrophobic methoxy group in HPMC allows it to act as a surfactant between the starch matrix/ air interface to strengthen gas bubbles in a gluten-free bread batter (Crockett and others 2011b).

## 5.5   CONCLUSIONS

Moisture content significantly affected the elastic and viscous moduli of potato starch-sorghum protein concentrate mixtures. At 55% db moisture content, the elastic and viscous moduli of sorghum protein concentrate obtained by extruding sorghum flour at 17% in-barrel moisture content were higher than the concentrate obtained by extruding sorghum flour at 32% in-barrel moisture content. Additionally, adding 10% of either 17 or 32 sorghum protein concentrate to potato starch resulted in the dough mixture having similar, or higher elastic and viscous moduli, than pure potato starch dough. In high moisture batter systems, such as the mixture with 65% db moisture content, the elastic and viscous moduli of mixtures containing protein concentrate were significantly lower than those of pure potato starch. This resulted possibly because the addition of sorghum protein concentrate weakened the starch gel and made it more fluid-like. Results of these fundamental rheological tests were useful in explaining the behavior of sorghum dough and batter food systems containing sorghum protein concentrate. The application of sorghum protein concentrates in tortilla dough showed that using protein concentrate 17 and adding 10% of protein concentrate improved the quality of sorghum tortillas. On the other hand, the addition of sorghum protein concentrates to sorghum batter-based bread was detrimental to its quality. Thus, this study was able to demonstrate that the nutritional quality of some gluten-free, starch-based foods can be improved without compromising quality.

## 5.6   ACKNOWLEDGEMENT

## 5.7   REFERENCES

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**Fig. 5.1 Frequency sweeps of sorghum protein concentrate dough (55% water) with potato starch. Sorghum protein concentrate levels were 0, 5 and 10% (based on starch). Evaluated at 0.0005 strain. A-Elastic modulus, G' and B-Viscous modulus, G".**
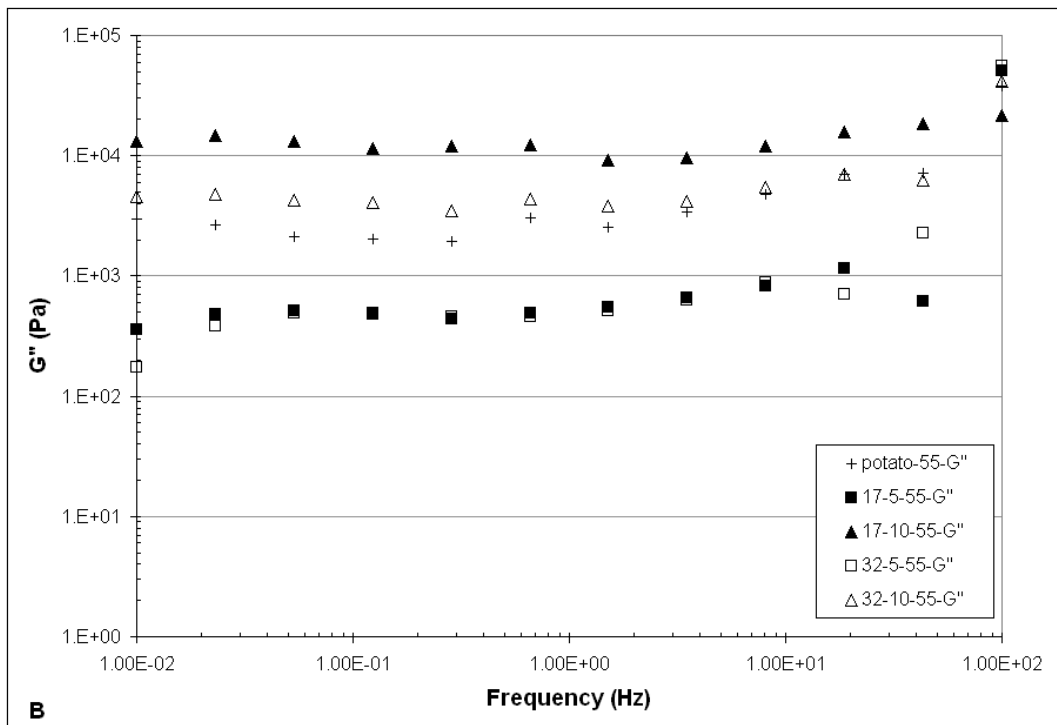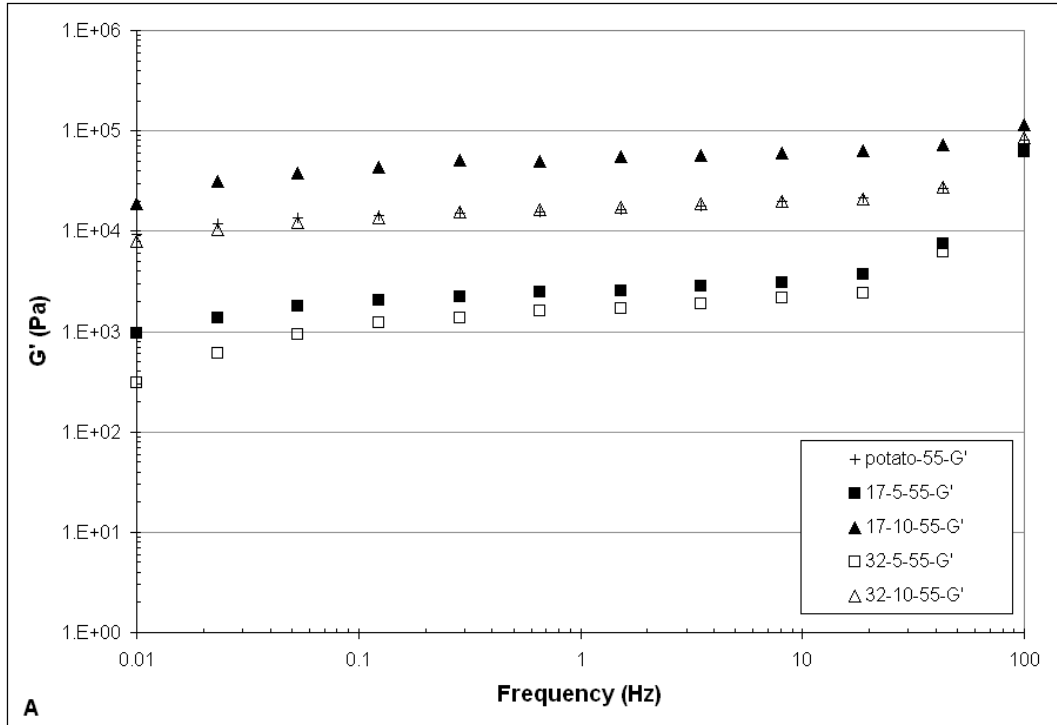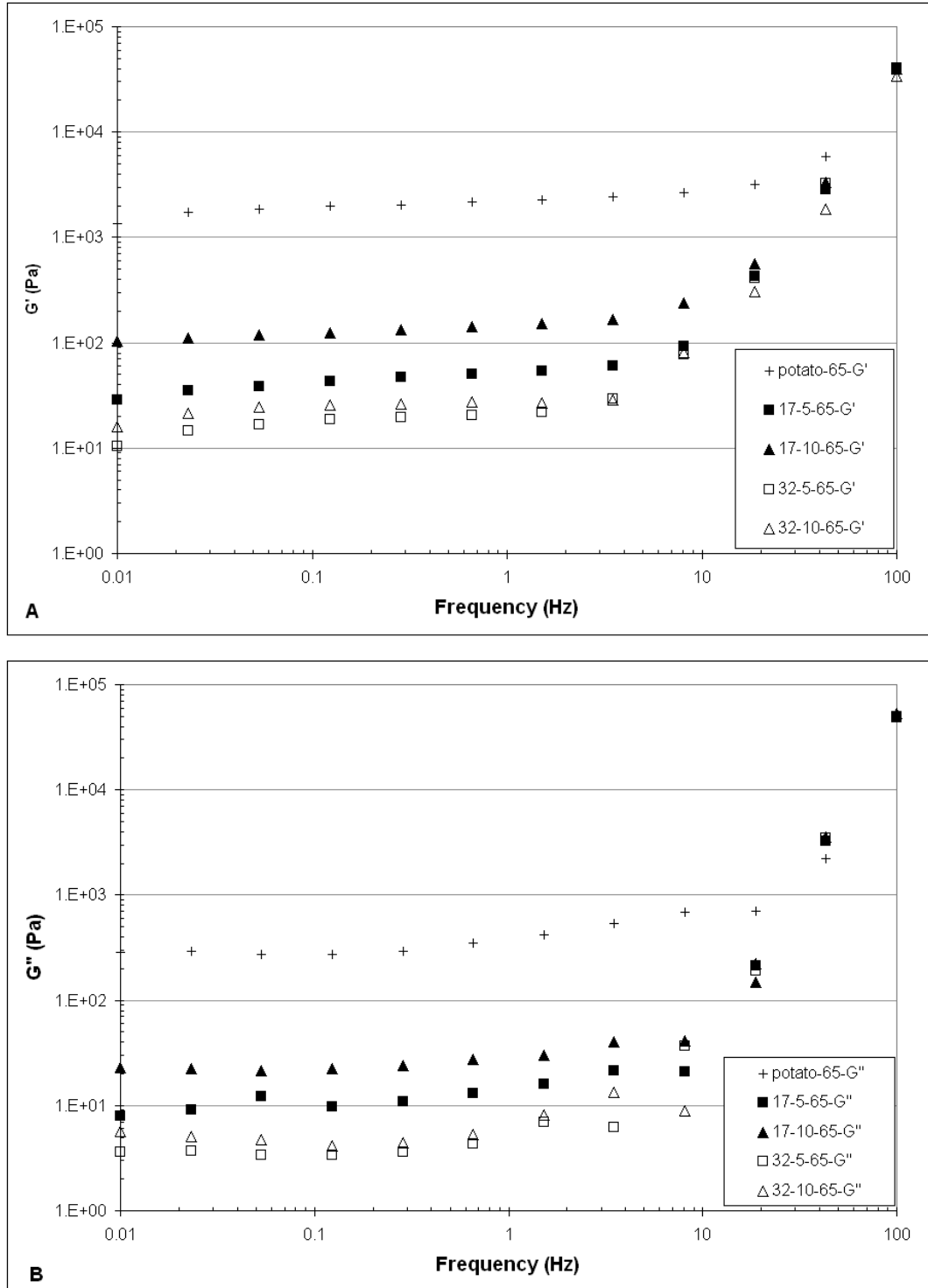
**Fig. 5.2 Frequency sweeps of sorghum protein concentrate batter (65% water) with potato starch. Sorghum protein concentrate levels were 0, 5 and 10% (based on starch). Evaluated at 0.005 strain. A-Elastic modulus, G' and B-Viscous modulus, G".**

**Table 5.1 Sorghum Tortilla Formulations**

| INGREDIENT | TREATMENTS[a] | | | | |
| --- | --- | --- | --- | --- | --- |
| | ADM | 17-5 | 17-10 | 32-5 | 32-10 |
| Sorghum flour | 100 | 100 | 100 | 100 | 100 |
| *% flour basis* | | | | | |
| Salt | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| Xanthan gum | 1 | 1 | 1 | 1 | 1 |
| Baking powder | 0.75 | 0.75 | 0.75 | 0.75 | 0.75 |
| Glycerin | 6.75 | 6.75 | 6.75 | 6.75 | 6.75 |
| Vegetable shortening | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 |
| Citric acid | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Sugar (granulated) | 15 | 15 | 15 | 15 | 15 |
| Monoglyceride | 2 | 2 | 2 | 2 | 2 |
| Sorghum protein concentrate | 0 | 5 | 10 | 5 | 10 |
| Water | 79 | 94 | 109 | 94 | 109 |

[a]Treatments with protein concentrates are labeled as type of protein concentrate-level of concentrate used.  17 and 32 are sorghum protein concentrates processed at 17 and 32% extrusion in-barrel moisture contents, respectively.

**Table 5.2 Sorghum Bread Formulations**

| INGREDIENTS | TREATMENTS[a] | | | | |
| --- | --- | --- | --- | --- | --- |
| | **Control** | **17-5** | **17-10** | **32-5** | **32-10** |
| sorghum flour, % of "flour" | 70 | 70 | 70 | 70 | 70 |
| potato starch, % of "flour" | 30 | 30 | 30 | 30 | 30 |
| "flour" | 100 | 100 | 100 | 100 | 100 |
| *% "flour" basis* | | | | | |
| salt | 1.75 | 1.75 | 1.75 | 1.75 | 1.75 |
| sugar | 1 | 1 | 1 | 1 | 1 |
| HPMC[b] | 2 | 2 | 2 | 2 | 2 |
| active dry yeast | 2 | 2 | 2 | 2 | 2 |
| Protein concentrate (% potato starch basis) | 0 | 5 | 10 | 5 | 10 |
| Water (% "flour" basis) | 105 | 108 | 110 | 108 | 110 |

[a]Treatments with protein concentrates are labeled as type of protein concentrate-level of concentrate used. 17 and 32 are sorghum protein concentrates processed at 17 and 32% extrusion in-barrel moisture contents, respectively.

[b]HPMC is hydroxypropylmethylcellulose.

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**Table 5.3 Means of Physico-chemical Properties for Protein Concentrate Type and Percentage Addition**

| Factors | Moisture Content (%)[c] | Specific Volume (cm³/g) | Rollability 2.5 cm | 1.5 cm | 1.0 cm | Extensibility Force (g) | Distance (mm) | Stretchability Force (g) | Distance (mm) |
|---|---|---|---|---|---|---|---|---|---|
| **Protein Concentrate[a]** | | | | | | | | | |
| 17 | 32.46 * | 0.288 | 5.0 | 4.8 | 4.9 * | 234 * | 29.85 * | 70 * | 3.95 * |
| 32 | 31.13 | 0.297 | 5.0 | 4.5 | 3.0 | 262 | 29.24 | 61 | 3.34 |
| **Percentage Addition[b]** | | | | | | | | | |
| 5 | 31.04 * | 0.288 | 5.0 | 4.4 | 3.5 * | 276 * | 29.76 * | 76 * | 3.97 * |
| 10 | 32.54 | 0.296 | 5.0 | 4.9 | 4.4 | 220 | 29.33 | 55 | 3.32 |

[a] 17 and 32 are sorghum protein concentrates processed at 17% and 32% extrusion in-barrel moisture contents, respectively.

[b] Percentages of protein concentrate added based on sorghum flour weight

[c] Asterisk denotes significant differences between types of protein concentrates or percentage addition, $p < 0.05$

**Table 5.4 Physico-chemical characteristics of sorghum tortillas**

| Sample | Moisture Content (%)[b, c] | Specific Volume (cm³/g) | Rollability 2.5 cm | 1.5 cm | 1.0 cm | Extensibility Force (g) | Distance (mm) | Stretchability Force (g) | Distance (mm) |
|---|---|---|---|---|---|---|---|---|---|
| No protein concentrate | 29.03 ± 0.20 a | 0.284 ± 0.012 a | 5.0 a | 4.0 ± 0.0 a | 1.5 ± 0.6 a | 360 ± 16 a | 29.84 ± 0.42 a | 99 ± 0 a | 4.22 ± 0.13 a |
| 17-5[a] | 31.75 ± 0.99 b | 0.284 ± 0.015 a | 5.0 a | 4.5 ± 1.0 a | 4.8 ± 0.5 b | 257 ± 25 b | 30.05 ± 0.27 a | 80 ± 6 b | 4.33 ± 0.35 a |
| 17-10 | 33.16 ± 1.02 b | 0.292 ± 0.012 a | 5.0 a | 5.0 ± 0.0 a | 5.0 ± 0.0 b | 210 ± 15 b | 29.66 ± 0.33 a | 61 ± 7 b | 3.57 ± 0.34 a |
| 32-5 | 30.33 ± 0.40 a | 0.293 ± 0.008 a | 5.0 a | 4.2 ± 0.5 a | 2.2 ± 0.5 a | 294 ± 18 b | 29.48 ± 0.23 a | 72 ± 8 b | 3.60 ± 0.29 a |
| 32-10 | 31.93 ± 1.10 b | 0.301 ± 0.016 a | 5.0 a | 4.8 ± 0.5 a | 3.8 ± 1.0 b | 229 ± 19 b | 29.00 ± 0.36 b | 49 ± 6 b | 3.07 ± 0.58 b |

[a]Protein concentrate-percentage addition(based on sorghum flour weight)

[b]Average of 2 replicates±standard deviation

[c]Different letters from the control (no protein concentrate) indicate significant difference, p<0.05

# Chapter 6- Conclusions and Recommendations

Sorghum protein concentrates can be produced by extrusion-enzyme liquefaction. While protein concentrates produced by batch liquefaction have only as much as 75% db protein content and 56% *in vitro* protein digestibility, protein concentrates produced by extrusion-enzyme liquefaction have much higher protein content (82% db) and digestibility (66%). At an optimal processing condition of 32% in-barrel moisture content with 2.5% fwb thermostable α-amylase added after extrusion, both high protein content and digestibility can be achieved (75% db and 65%, respectively). Extruding sorghum flour under high shear conditions (such as reducing in-barrel moisture content to 17%) resulted in higher protein content and improved functionality. In addition to having higher protein content and digestibility, the advantages of using extrusion-enzyme liquefaction over batch liquefaction included using less process water and potentially reduced processing time and larger throughput. Moreover, extrusion-enzyme liquefaction used food-compatible reagents, which cannot be said for other methods of producing sorghum protein concentrate. The addition of sorghum protein concentrates produced by extrusion-liquefaction to tortilla dough was successful in improving sorghum tortilla quality. Sorghum tortilla containing sorghum protein concentrates were softer and more rollable than those without the concentrates.

Extrusion-enzyme liquefaction of sorghum protein concentrate is a high throughput process with commercial promise. Future work includes scale-up to a commercial level. In doing so, factors such as grain selection would be an important parameter to study. On the one hand, hard sorghum may be better than soft sorghum because milling the former would result in greater starch damage, which would lead to more extensive starch liquefaction and a purer protein concentrate. On the other hand, hard sorghum, having a larger vitreous endosperm portion, would have more cross-linked proteins (Ioerger and others 2007), which did not appear to be desirable based on the results of this work (Chapter 5). However, there may be some applications where cross-linked proteins can be utilized. Using waxy sorghum (100% amylopectin) may also be a better starting material than normal sorghum. In waxy sorghum, starch granules and the protein matrix around it are more digestible (Rooney and Pflugfelder 1986). Waxy starch granules were shown to have microscopic holes (Yan 2011), which also explains its susceptibility to α-amylase digestion. Additionally, tannin-free sorghum should be

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used in the liquefaction process because tannin is known to form indigestible complexes with sorghum protein (Duodu and others 2003) and α-amylase (Wu and others 2007), both of which interfere with liquefaction efficiency. The use of high lysine sorghum varieties can also be explored as sorghum is typically poor in lysine. On the other hand, sorghum is rich in leucine (Klopfenstein and Hoseney 1995).

Another factor to consider in commercial scale-up of the extrusion-enzyme liquefaction process is the method of milling. Debranning needs to be performed prior to milling in order to obtain a concentrate with high protein content. Pin-milling at high speed is probably the most desirable method for this type of process. This method of milling was found to yield very fine flour with considerable starch damage (Frederick 2009).

In the extrusion process, other ways to increase mechanical energy need to be explored. For example, a more aggressive screw configuration can be used. Alternatively, the addition of thermostable α-amylase in the extruder can again be explored but under prolonged residence time. The screw configuration or die diameter can be changed to increase residence time in the extruder. The use of enzyme concentrations below 2.5% fwb also need to be explored. Batch liquefaction typically uses 0.04 to 0.84% fwb of the thermostable α-amylase (Zhao and others 2008). High levels of this enzyme were used in this study in order to compensate for the damaging effects of the extrusion process. However, in hindsight, it would have been prudent to also evaluate the use of the same enzyme levels typically used in batch liquefaction.

When conducting extrusion-enzyme liquefaction experiments using the laboratory-scale extruder in Chapter 3, we saw huge variability in specific mechanical energy (SME), which translated to large differences in protein content and digestibility for the same set of process conditions. The identifiable sources of error follow. First, treatments with the enzyme preparation tend to be sticky and lumpy, which resulted in variations in feed rate. This was further complicated by the fact that the feeder screw of the laboratory-scale extruder is volumetric and is, thus, affected by variations in feed density and amount of feed loaded. We presently have no mechanism to accurately control this. Second, the laboratory-scale extruder used in this study is designed for extruding plastics and not food. For this reason, torque readings and computed SME values were very large. Third, because the laboratory-scale extruder is also very sensitive to very slight variations in moisture content and feed composition, the slightest differences in weight measurements were magnified.

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In experiments wherein thermostable α-amylase was added together with sorghum flour in the extruder, we observed that SME dropped significantly and that extrudate consistency became thinner. While these phenomena can be a result of enzymatic action, we were unable to isolate the effect of sugar in the enzyme preparation. Sugar was used as a diluent and stabilizing agent (to improve thermostability) by the enzyme manufacturer and it made up a considerable portion of the enzyme preparation. The exact percentage of sugar cannot be declared for proprietary reasons. Since sugar is known to reduce SME (Barrett and others 1995; Hsieh and others 1990), experiments isolating the effects of sugar can be conducted. By testing for reducing sugars (Fehling's and/or Dinitrosalicylic acid Test) before and after extrusion, one can determine the extent of enzymatic action in the extruder and whether sugar was indeed the primary reason for reduction in SME.

Although initial investigations in the laboratory indicated that liquefaction of extruded sorghum flour is faster than raw sorghum flour, a more elaborate experiment needs to be designed and conducted to evaluate enzyme dosage and the length of time needed to complete liquefaction. This experiment would require the evaluation of the quantity of total reducing sugars and dextrose equivalent at several stages of processing. Information gathered from these experiments can be used to conduct a much needed economic analysis that would show the concrete financial advantage of adding an extrusion step to the liquefaction process. Financial data can then be used to support the feasibility of commercial scale-up operations.

The sorghum protein concentrates need to be further characterized. Confocal scanning laser micrographs (CLSM) of protein concentrate 17 and 32 need to be done in order to understand differences in functionality. In Chapter 3, CLSM was found to be a useful tool in illustrating the link between disaggregation and *in vitro* protein digestibility. CLSM can be used to confirm the *in vitro* protein digestibility of the concentrates by looking at the extent of protein disaggregation. Protein concentrate 17 is expected to show more protein disaggregation than concentrate 32 because it (17) is more digestible. CLSM can also be done on the starch-protein concentrate doughs prepared for rheological characterization in Chapter 5. This information would reveal whether or not sorghum proteins formed a network or remained in the dispersed phase.

In Chapter 4, we saw that increasing enzyme concentration led to a reduction in the protein digestibility of concentrate 17. We suggested that liquefaction was completed sooner at

higher enzyme concentration, which left more time for soluble starch to form indigestible complexes with the disrupted sorghum proteins.  Possibly, bonds were created between soluble starch and the hydrophilic residues of the proteins, which were exposed as a result of the shearing action in the extruder.  This reaction was more apparent with protein concentrate 17 than with concentrate 32 because the former was exposed to higher specific mechanical energy.  Future work can be done to test the hypothesis that indigestible complexes of soluble starch and protein formed during liquefaction.  One way to test for the formation of starch-protein complexes is to compare the starch content of the protein concentrate using the standard total starch test with and without dimethyl sulfoxide (DMSO) pre-treatment.  If starch content with DMSO is larger than that without DMSO, then soluble starch may have truly formed complexes with sorghum protein.

Other applications for these concentrates can be tested.  For example, the emulsifying properties of protein concentrate 17 and 32 can be compared.  Given that concentrate 17 is more digestible than concentrate 32, it is possible that more of its peptides have exposed hydrophilic and hydrophobic ends, which would make it a better emulsifier than protein concentrate 32.  Even though kafirins are hydrophobic in their unaltered state, perhaps their disruption and disaggregation as a result of extrusion exposed hydrophilic residues, which could enable sorghum proteins to have emulsifying properties.  Potential applications for sorghum protein concentrates also include in gluten-free pasta, crackers, and extruded breakfast cereal.

Protein modifications can be done on the sorghum protein concentrates to improve functionality.  First, the concentrates have to be further purified to at least 90% db protein content.  By purifying the concentrates, true protein functionality can be evaluated and further protein modification steps can be more productive.  Extraneous material can interfere with any attempts to modify sorghum proteins.  The method of modification will depend on the desired application.  For example, if sorghum proteins were to be used as an emulsifier, proteases can be used to reduce peptide size and to expose both hydrophobic and hydrophilic ends.  Chemicals such as sodium metabisulfite, cysteine and glutathione can also cleave sorghum proteins.  However, one has to also consider the safety and cost of these chemicals prior to use.  On the other hand, transglutaminase or oxidases can be used to cross-link proteins if gluten-like viscoelasticity is desired.  Then again, modifying proteins with transglutaminase should be exercised with caution as this may not be safe for gluten-free foods.

In conclusion, extrusion-enzyme liquefaction was successful in producing sorghum protein concentrates with improved protein content and digestibility. While we have proven that these sorghum protein concentrates have potential use in gluten-free foods, more characterization and application studies are needed. Opportunities also exist for process improvement as well as modification of sorghum protein properties.

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