IMPACT OF SORGHUM PROTEINS ON ETHANOL FERMENTATION AND
INVESTIGATION OF NOVEL METHODS TO EVALUATE FERMENTATION QUALITY

by

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B.S., Henan University of Technology, China, 1992
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Biological and Agricultural Engineering
College of Engineering

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2008
Abstract

Sorghum has been considered one of the best species dedicated to biofuel production because of its drought tolerance, low fertilizer or pesticide input, established production systems, and genetic diversity. The mission of this research was to better understand the relationship among “genetic-structure-function-conversion.” The main focus of this research was to study the impact of sorghum proteins on ethanol fermentation and to investigate novel methods for evaluation of sorghum fermentation quality.

Changes of sorghum protein in digestibility, solubility, and microstructure during mashing were characterized. Sorghum proteins tended to form highly extended, strong web-like microstructures during mashing. The degree of protein cross-linking differed among samples. Formation of web-like microstructures due to cross-linking reduced conversion efficiency. A rapid method for extracting proteins from mashed and nonmashed sorghum meal using sonication (ultrasound) was developed, with which the relationships between the levels of extractable proteins and ethanol fermentation properties were determined. There was a strong relationship between extractable proteins and fermentation parameters. Ethanol yield increased and conversion efficiency improved significantly as the amount of extractable proteins increased. The Rapid-Visco Analyzer (RVA) was used to characterize pasting properties of sorghum grains. Results showed a strong linear relationship between ethanol yield and final viscosity, as well as setback. A modified RVA procedure (10 min) with an application of α-amylase was developed to simulate the liquefaction step in dry-grind ethanol production. There was a remarkable difference in mashing properties among the sorghum samples with the normal dosage of α-amylase. The modified RVA procedure is applicable not only for characterization of mashing properties but also for prediction of tannin content and for optimization of α-amylase doses for starch liquefaction. A small-scale mashing (SSM) procedure requiring only 300 mg of samples was investigated as a possible method of predicting ethanol yield of sorghum grain. There was a strong linear correlation between completely hydrolyzed starch (CHS) from SSM and ethanol yields from both traditional and simultaneous saccharification and fermentation procedures. CHS was a better indicator for predicting ethanol yield in fermentation than total starch.
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Dedication

This work is dedicated to my beloved family.
CHAPTER 1 - INTRODUCTION

GENERAL BACKGROUND

Ethanol’s chemical properties make it very useful for some applications, especially as an additive in gasoline. It serves as an oxygenate to prevent air pollution from carbon monoxide and ozone; as an octane booster to prevent early ignition or “engine knock”; and as an extender of gasoline stocks. In purer forms, it can also be used as an alternative to gasoline in automobiles specially designed for its use.

Promotion of alternatives to petroleum, including fuel ethanol, has been an ongoing goal of the U.S. energy policy. Federal incentives for ethanol use-including tax incentives, the reformulated gasoline oxygenate standard, and the renewable fuels standard (RFS)-have promoted significant growth in the ethanol market. Ethanol production in the U.S. is now undergoing unprecedented expansion. In 2006, a record 4.9 billion gallons of ethanol were produced from 110 biorefineries located in 19 states across the country, which exceeded the previous year’s record of one billion gallons by more than 25% (Renewable Fuels Association, 2007). However, this figure represents only 3.6% of the approximately 140 billion gallons of gasoline consumed in the same year. Domestic production capacity continues increasing to meet the growing demand, including increased demand resulting from implementation of the RFS established by the Energy Policy Act of 2005. At the beginning of 2007, total existing production capacity was approximately 5.6 billion gallons per year and is expected to grow to 11.7 billion gallons per year by 2009, counting existing plants and plants under construction. The production capacity is well in excess of the 7.5 billion-gallon supply required in 2012 by the RFS.

Researchers and ethanol producers have shown that grain sorghum is a reasonable feedstock (technically acceptable, fits the infrastructure, and can be economically viable) for ethanol and could make a larger contribution to the nation’s fuel ethanol requirements. Interest in using grain sorghum for bio-industrial applications is now growing in the U.S. (Farrell et al., 2006). Although currently only about 2.5% of fuel ethanol is produced from grain sorghum, annual consumption of sorghum by the ethanol industry is steadily increasing from 11.3% in 2004 to 15% in 2005, and to 26% in 2006. Due to climate diversity and continuing decline of
water resource, utilization of our dry land to grow grain sorghum is critically important to insure our sustainable economic development and rational economic distribution.

Starch and protein are two major components in sorghum grain. Current research in our laboratory demonstrated that starch content is a good indicator of ethanol yield in the dry-grind process, but starch content itself could not explain conversion efficiency well (Wu et al., 2007). Sorghum protein content varies from 6 to 18%, with storage proteins generally comprising 70-90% of total protein (Lookhart et al 2000). Sorghum is rich in potential nitrogen for yeast growth during fermentation. However, a significant problem with sorghum is its comparably poor nutritional quality. Protein digestibility in wet-cooked sorghum was considerably lower when compared with other cereals (Maclean et al 1981; Hamaker et al 1987). It is not surprising that this problem will become more severe when sorghum is used to produce fuel ethanol, because fermentation condition is more temperate than in an in vitro pepsin digestion environment. Yeast can not use complex nitrogenous materials for growth unless they are first hydrolyzed to simple amino acids, dipeptides, or perhaps tripeptides (Berry and Brown 1987). Growth media are often supplemented with complex mixtures of amino acids.

Nevertheless, the effect of sorghum protein on ethanol fermentation is far beyond nutrition deficiency. Among the cereals, sorghum grain generally has the lowest starch digestibility due to interactions between protein and starch, which may reduce the susceptibility of native and gelatinized starch to enzyme hydrolysis (Rooney and Pflugfelder 1986). Sorghum grains with lower capacities for starch gelatinization were observed to have more kafirin-containing protein bodies, which may restrict the starch granules from full gelatinization, thereby resulting in lower digestibility (Chandrashekar and Kirleis 1988). Neither the starch itself nor the outer layer materials of sorghum seeds appeared to be related to poor starch digestibility, and treating flour with pepsin before cooking or cooking with a reducing agent led to an increase in starch digestibility, suggesting that protein may act as a barrier to starch digestion (Zhang and Hamaker 1998). Mashing, including liquefaction and saccharification, is an absolutely necessary step when sorghum is used to produce fuel ethanol. Thus endosperm proteins in sorghum may play an important role in determining ethanol yield and conversion efficiency by impairing complete enzymatic digestion of sorghum starch to fermentable sugars. The role of protein in sorghum grain has been ignored and research activities about the impact of sorghum protein on ethanol fermentation have been limited in the past.
Fuel ethanol is now produced from corn by either the dry-grind (82%) or wet-mill (18%) process, and the majority of those biorefineries being constructed are dry-grind plants (Renewable Fuels Association 2007). At present, wet-milling of grain sorghum is rarely done industrially because of the difficulty in complete recovery and purification of starch from sorghum, and the off-color characteristics of sorghum starch (Yang and Seib 1995). Moreover, yield of oil is low from grain sorghum because of the small size of the germ and low proportion of germ in the kernel when contrasted to wet-milling of corn (Watson 1984). Therefore, sorghum, as an alternative feedstock in replacing corn, is inevitably utilized in the dry-grind process to produce fuel ethanol. Among the five basic steps in the conventional dry-grind ethanol process are grinding, cooking, liquefaction, saccharification, and fermentation. Cooking goes on throughout the entire process, beginning with mixing the grain meal with water (and possibly backset stillage) to delivery of a mash ready for fermentation. Mashes used in industry for production of fuel ethanol usually have a dissolved solids content in the range of 20-24 g/100 mL of mash, and normally a grain-to-water ratio of 1:3 is used (Thomas et al 1995). More recently fuel alcohol plants run at alcohol levels as high as 19-20% by volume, the average being nearer to 16-17% by volume (Kelsall and Lyons 2003). Therefore, viscosity is extremely high following gelatinization. In dry-grind processing, thermostable α-amylase enzymes are added during cooking as thinning agents, which bring about reduction in viscosity and partial hydrolysis of starch. Retrogradation of starch is thus avoided during subsequent cooling. Lower mash viscosity improves heat transfer efficiency in the heat exchangers and allows the plant to process higher levels of dry solids, which gives a significant energy reduction in steam heating of the mash and cooling the cooked liquefact prior to fermentation. Grain sorghum is similar to corn in composition, kernel structure, starch properties (Watson 1984), and ethanol yield (Wu et al 2007). Adoption of sorghum in replacing corn to produce fuel ethanol has been very successful, but there are still concerns regarding difficulties with handling this cereal in ethanol production. In a recent study (Wu et al 2007), a high-tannin sorghum sample was reported to have difficulty in liquefaction with an abnormal high viscosity when compared with normal non-tannin samples. If such problems occur in a dry-grind mill, the plant will have to reduce the contents of dry solids, strengthen the agitation, or increase the amylase levels in the mash, all of which will increase operating costs. Thus, mashing property can be regarded as an important quality trait of sorghum grain in respect to ethanol fermentation. It is necessary and important for scientists to
characterize the mashing properties of different genotypes and develop new or modified cultivars, which could be easily handled in the dry-grind industry. It will be beneficial for ethanol plants to develop quick methods to predict the fermentation quality of grain sorghum. However, information about mashing properties of sorghum grain is quite limited.

With hundreds of sorghum hybrids used in the commercial channel, there is a large variation in fermentation quality among these hybrids. Thus it is important for the ethanol industry and sorghum producers to have proper methods to predict their ethanol yields as well as conversion efficiencies. Much fewer researchers have reported about the fermentation quality of sorghum in comparison with other grains like corn, wheat, and barley. Little research has been conducted to develop methods for the evaluation of sorghum fermentation quality.

Among the cereal crops, sorghum has received the least amount of attention with regard to the relationship between genotype, grain composition, and end-use quality for fuel ethanol. To better understand such inter-relationships, additional research on the detailed composition of sorghum grain and bioprocessing results is required.

RESEARCH OBJECTIVES

The overall mission of this research is to better understand the relationship among genotype, grain composition, and end-use quality of sorghum grain for fuel ethanol. On the basis of knowledge on the inter-relationships between compositional characteristics and bioprocessing results, novel methods are expected to be investigated for the evaluation of sorghum fermentation quality. The research approach will include the following key tasks:

1) To investigate the relationship between fermentation parameters and characteristics of sorghum protein including protein content, available nitrogen as food by yeast, protein digestibility, extractability, and microstructure.

2) To characterize the mashing properties of sorghum grain during enzymatic hydrolysis.

3) To investigate new methods to evaluate fermentation quality of sorghum grain, such as ethanol yield, conversion efficiency, mashing properties, etc.

RELATED CURRENT AND PREVIOUS RESEARCH
Proteins in Sorghum

Proteins can be classified by function (metabolic, storage, or structural proteins) or solubility (Hoseney 1994). The Osborne classification divides proteins into four basic groups based on their solubilities: albumins (soluble in water), globulins (soluble in dilute salt solutions), prolams (soluble in alcohol), and glutelins (soluble in dilute acids or bases). Albumins and globulins are primarily physiologically active proteins, while prolams and glutelins are storage proteins. The prolams in sorghum are called kafirin. Sorghum grain varies in protein content from 6 to 18%, with storage proteins (kafirins) generally comprising 70-90% of the total protein (Laszity 1984; Lookhart et al 2000). Sorghum is rich in potential nitrogen for yeast growth during fermentation.

Using the solubility-based classification scheme, Jamunathan et al (1975) divided sorghum proteins into albumins, globulins, kafirins, cross-linked kafirins, and glutelins. The kafirins have been further classified as α- (Mr 25 and 23 kDa), β- (Mr 20, 18, and 16 kDa), and γ- (Mr 28 kDa) groups on the basis of solubility, molecular weight, and structure (Shull et al 1991). In sorghum, kafirin contents are 68-73% of protein in whole grain and 72-82% of protein in endosperm (Hamaker et al 1995). Alpha-kafirin represents 66-71% and 80-84% of the total kafirins in the opaque and vitreous kernel sections, respectively; β-kafirin, 10-13% and 7-8%; and γ-kafirin, 19-21% and 9-12% (Watterson et al 1993). Protein bodies are well-defined structures in the sorghum endosperm and contain the kafirin proteins. Internal protein body structure is such that γ-kafirin and β-kafirin encapsulate α-kafirin in a disulfide-bound polymeric network (Shull et al 1992). Within the protein body, the kafirins are distributed in a non-homogeneous fashion. Alpha-kafirin is located in the light-staining regions, mainly in the interior of the protein body; and β- and γ-kafirin are found in the dark-staining areas inside, as well as at the periphery of the protein body (Shull et al 1992). A developmental study showed that the decrease in protein digestibility in maturing sorghum grain is due to the drying effect and the parallel increase in formation of disulfide-bound complexes involving β- and γ-kafirin (Duodu et al 2002). In uncooked sorghum, various disulfide-bound protein oligomers comprising γ- (Mr 29 kDa), α1- (Mr 26 kDa), and α2- (Mr 24 kDa) kafirins and polymers containing β- (Mr 18 kDa) as well as γ- and α1-kafirins were found (El Nour et al 1998). It was hypothesized that γ-, and to a lesser extent β-kafirins, form a disulfide-bound enzyme-resistant layer at the periphery of the protein bodies that restricts access by proteases to easily digestible α-kafirin (Oria et al 1995a).
Sorghum in the uncooked state has been reported to have a lower digestibility when compared to maize and other cereal grains such as rice and wheat (Mertz et al. 1984; Duodu et al. 2002). Several studies suggest that it is the nature of the proteins within the protein bodies that is mostly responsible for low protein digestibility of sorghum grain. It was revealed that kafrins are the last proteins to be digested (Hamker et al. 1986), and that the addition of a reducing agent improved their uncooked and cooked digestibility (Oria et al. 1995a, 1995b). These studies support the ideas that structural features within the proteins, particularly disulfide bonds, negatively influence protein hydrolysis. Alpha-kafrin, although easily digested when isolated from protein bodies in its native unreduced form, was digested only after γ- and β-kafrins when flour was used for in vitro digestion. These results, plus a scanning electron microscopy study (Rom et al. 1992), indicate that the breakdown of sorghum storage proteins starts on the outside of protein body and progress toward the interior. Low digestibility of sorghum prolamins has been shown to be related to tannin content (Oria et al. 1995b). However, even among the low-tannin or tannin-free cultivars, low protein digestibility still exists (Elkin et al. 1996).

**Effect of Wet-cooking on Sorghum Protein Digestibility**

Partially denatured proteins are more digestible and have better foaming and emulsifying properties than do native proteins (Damodaran 1996). Thermal denaturation of trypsin inhibitors in legumes markedly improves digestibility and biological availability of legume proteins. Farag (1999) suggested that processing sunflower meal by either heating or autoclaving treatment for 20 min improves in vitro protein digestibility through decomposing the chlorogenic acid.

Upon wet-cooking, as in porridge making, the digestibility of sorghum proteins decreases substantially compared to that of maize. This observation has been made both in human studies (MacLean et al. 1981) and in vitro protein digestibility (Rom et al. 1992; Oria et al. 1995b; Duodu et al. 2002). The reduction in protein digestibility of cooked sorghum has generally been attributed to the formation of cross-linked protein polymers, which are resistant to proteolysis (Hamaker et al. 1987, 1994; Rom et al. 1992; Oria et al. 1995b; Duodu et al. 2002; Nunes et al. 2004). In vitro protein digestibility thus became an important marker of protein cross-linking. Use of sodium bisulfite as a reducing agent during cooking did not completely eliminate the problem of lowered sorghum protein digestibility (Oria et al. 1995b). The observation that the digestibility was not fully reversed to the level of uncooked flour may be due to the presence of
disulphide bonds inaccessible to the reducing agent (Oria et al 1995b). The resistance to reduction of protein oligomers in cooked sorghum may be because they are in a conformation that does not allow the reducing agent easy access to disulfide bonds (Duodu et al 2002).

Disulphide cross-linking of proteins is also found in maize during wet-cooking, but the digestibility of maize is not reduced to the same extent as that of sorghum (Battman-Azcona et al 1998; Duodu et al 2002). It appears that more disulphide-bonded oligomers are formed in sorghum during cooking compared to maize, which may in part explain the lower digestibility of sorghum proteins (Duodu et al 2002). The small differences in the amount of disulphide bonds formed cannot completely explain the differences in reduction in digestibility between wet-cooked sorghum and maize (Duodu et al 2003).

The inability to explain the observed difference in digestibility with maize appears to be a shortcoming of the disulphide-bonding hypothesis. The possibility of non-disulphide cross-links involving reduction in protein digestibility of cooked sorghum has been proposed (Duodu et al 2003). High-molecular-weight kafirin oligomers with $M_r$ 45-60 kDa (Duodu et al 2002; Nunes et al 2004) have been identified in wet-cooked sorghum, and in the pepsin indigestible residues from cooked sorghum. These oligomers were not completely cleaved by a reducing agent, also suggesting that disulphide bonding may not be the only reason for the reduction in sorghum protein digestibility with wet-cooking.

Duodu et al (2001) studied the changes brought about in the secondary structure of sorghum and maize proteins by wet-cooking using FTIR and $^{13}$C NMR spectroscopy, and found that during wet-cooking, sorghum and maize proteins undergo a change in secondary structure from an alpha-helical to anti-parallel, intermolecular beta-sheet conformation. It is possible that the change to beta-sheet conformation could encourage the formation of disulphide cross-links between polypeptides in close proximity, resulting in a rigid, less digestible structure. Changes in sorghum appear to occur to a slightly greater extent compared with maize. However, it is unlikely believe that these small structural differences could alone account for the large decreases in sorghum protein digestibility (Duodu et al 2003).

Actually mashing is a kind of wet-cooking process. The main difference between mashing and wet-cooking is that mashing completely converts starch into fermentable sugars, while wet-cooking only gelatinizes starch granules. Although heat-stable alpha-amylase did not affect the protein digestibility of cooked sorghum, enzymatic treatment significantly improved
the kafrin extractability of the cooked sorghum flour (Oria et al 1995b). However, treating cooked sorghum and maize whole grain and endosperm flours with alpha-amylase to reduce sample complexity before pepsin digestion did improve protein digestibility (Duodu et al 2002). It looks like cross-linking is the main change during mashing of sorghum proteins. The information about the effect of mashing on sorghum proteins is limited, but those discoveries originated from the studies on sorghum protein changes during wet-cooking could be applied to partially explain what happens to sorghum proteins by mashing; for example, reduction in solubility, decrease in digestibility, etc.

**Applications of the Rapid Visco Analyzer (RVA) in Grain Sorghum**

The RVA was originally designed to rapidly test for sprout damage in wheat during receival (Ross et al 1987). Additions of linearly ramped heating and cooling abilities to the RVA expanded its application to include starch pasting tests through the classic heat-hold-cool profile (Wrigley et al 1996). Few works have been reported in the area of using the RVA to study grain sorghum and its associated products when compared with wheat, rice, and corn. Most researchers used the RVA to investigate the pasting properties of isolated sorghum starches (Moheno-Perez et al 1997; Beta et al 2000; Xie and Seib 2000; Beta et al 2001; Beta and Corke 2001a, b; Agu et al 2006) or starches in raw (Cruzy Celis et al 1996; Taylor et al 1997; McDonough et al 1998; Hugo et al 2000; Agu et al 2006), dehulled (Taylor et al 1997), decorticated (Cruzy Celis et al 1996; Suhendro et al 2000), flaked (Cruzy Celis et al 1996; McDonough et al 1998), and malted (Hugo et al 2000) sorghum grains, with solid levels ranging from 8.6 to 14.0% (w/w) in the slurries. Among the varied RVA procedures were 13-, 18-, and 22-min temperature profiles reported in the above mentioned literature. Beta et al (1995) assayed α-amylase in sorghum malts by measuring the decrease in viscosity using the RVA with a 3-min rapid-pasting test. In order to simulate an industrial mashing process, Goode et al (2005) successfully used the RVA as a rheological tool to characterize the effects of different ratios of malt-to-barley adjunct. Clear correlations were found between the level of barley adjunct and RVA parameters. Agu et al (2006) showed that the RVA is useful in assessing wheat for production of grain whisky, and they found that both the RVA peak and final viscosities were highly correlated with alcohol yield.
Factors Impacting Sorghum Fermentation Quality

It was reported that variety, ratio of amylose to amylopectin, protein-starch interaction, tannin level, mash viscosity, formation of amylose-lipid complexes, particle size of ground sorghum meal, etc. had significant effects on ethanol yield and conversion efficiency from grain sorghum (Zhan et al 2003; Wu et al 2007). Starch content in grain sorghum could range from 64 to 74%, which could result in a 22% difference in ethanol yield for the same amount of grain. Ethanol yields from sorghums with similar starch contents varied as much as 7.4%, which indicated that not all the starch equally contributes to ethanol yield (Wu et al 2007). Waxy and heterowaxy varieties generally have higher fermentation efficiencies than non-waxy varieties, because amylose is likely to form amylose-lipid complexes in seeds or during mashing, which are resistant to enzymatic hydrolysis (Wu et al 2006b).

As in other cereal grains, protein content in grain sorghum is inversely proportional to starch content, and thus shows a negative effect on ethanol yield (Zhan et al 2003; Wu et al 2007). Ethanol yield could show as much as an 8% difference for sorghum varieties with similar protein content. The most probable reasons for the adverse effects of protein on ethanol fermentation could be the formation of a web-like protein matrix by crosslinking of sorghum proteins during mashing or cooking, which prevented the starch granules in the enmeshed matrix from gelatinization and limited their accessibility to enzyme hydrolysis, and consequently lowered the digestibility of sorghum starch (Zhang and Hamaker 1998; Duodo et al 2003; Wu et al 2007).

Adverse effects of tannin on digestibility of sorghum protein and starch have long been recognized. Tannins are well known for their adverse effect on starch digestibility because of their ability to interact with proteins (including hydrolytic enzymes), metal ions, and polysaccharides (Davis and Hoseney 1979; Deshpande and Salunkhe 1982; Rooney and Pflugfelder 1986; Schofield et al 2001). Wu et al (2007) reported that liquefaction of starch in tannin sorghums was more difficult and slower than in normal and waxy sorghums. Tannins in sorghum retarded the hydrolysis process and resulted in viscous mash, which could not be solved by simply increasing amylase usage during mashing. The adverse effect of tannins on conversion efficiency was also confirmed.

Research has also indicated that the growing environment greatly affected physical properties and chemical compositions of cereal grains (Beta and Corke 2001; Tester and
Karkalas 2001) and their application attributes (O’Brien and Orth 1977; Mikhaylenko et al 2000; Swanston et al 2005). Swanston et al (2007) reported that environmental conditions had significant influence on ethanol yield of wheat varieties in the UK. Zhan et al (2003) found that the effect of genotype and environment on ethanol production is related to both chemical composition and physical properties of sorghum grain samples.

Current Methods for Evaluation of Fermentation Quality

Ethanol yield and conversion efficiency are the two most important quality traits of cereal grains when they are used to produce fuel ethanol. It is no doubt that laboratory fermentation is the most direct and reliable method to evaluate fermentation qualities of cereal grains, but currently reported fermentation procedures are time consuming and tedious. In addition, they require relatively large quantity of samples.

Previous research by our group has shown that starch content in sorghum is a good indicator of ethanol yield in the dry-grind process (Zhan et al 2003; Wu et al 2007). There is no significant relationship between starch content and conversion efficiency (Wu et al 2007). Therefore, starch content cannot be used to predict conversion efficiency of grain sorghum.

Starch determination methods are broadly grouped into acid hydrolysis or enzymatic procedures. Acid hydrolysis procedures can only be applied to pure starch samples and thus have limited application. Enzymatic procedures vary in pre-treatment steps, starch gelatinization, liquefaction and dextrinization, hydrolysis of dextrins to glucose, and glucose measurement. In a currently used starch assay procedure (AACC International 2000), starch is hydrolyzed by sequential treatment with thermostable $\alpha$-amylase and amyloglucosidase. This technology requires high-purity enzymes, especially amyloglucosidase free of contaminating activities of cellulase and catalase. Cellulase contamination contributes to false high starch values because of cellulose hydrolysis, and catalase reduces the stability of the chromogen formed in glucose assay methods based on use of the GOPOD reagent (McCleary et al 1997). Megazyme has produced, and now offers a total starch assay kit based on use of thermostable $\alpha$-amylase and amyloglucosidase. However, widespread use of this approach in industry has been limited by the prohibitive cost of per sample analysis.

According to Approved Method 76-13 (AACC International 2000), for samples containing enzyme-resistant starch, complete solubilization and dextrinization requires pre-
treatment with dimethyl sulphoxide (DMSO) at boiling. Results on sorghum samples using Megazyme kits found that total starch content with DMSO pre-treatment was slightly higher than its counterparts without DMSO (data unpublished). For the purpose of avoiding underestimation of starch content in sorghum samples, pre-treatment with DMSO is favorable and necessary. However, it is impossible for ethanol fermentation practices to have a DMSO pre-treatment before liquefaction and saccharification, which could be one of the reasons that total starch content only explained 78% of the variability of ethanol yield among 70 sorghum samples (Wu et al 2007). To completely convert starch to glucose in a short time required enzyme levels much higher than those used in ethanol fermentation for the quantified amount of substrates, which could be another reason that total starch content failed to well explain the variability of ethanol yield among sorghum samples. Total starch assay is also time consuming and complicated, requiring too many manipulations. It has unsatisfactory repeatability and reproducibility, requiring many replications to achieve ideal results. Overall, there are several disadvantages in using total starch content to predict ethanol yield of grain sorghum.

Research from Pioneer Company has shown that the high total fermentables (HTF) trait of corn is a more accurate indicator of dry-grind ethanol production than total starch (Bryan 2003). Additionally, Pioneer has developed a point-of-sale assay using whole grain near-infrared (NIR) technology that allows ethanol plants to predict the value of corn for ethanol production by identifying HTF grain arriving at the plant.

LITERATURE CITED


Chemistry and Technology. R. L. Whistler, E. H. Psachall, and J. N. BeMiller, eds.
Academic Press: Orlando, FL.


Wrigley, C. W., Booth, R. I., Bason, M. L. and Walker, C. E. 1996. Rapid Visco Analyser:
Progress from concept to adoption. Cereal Foods World 41:6-11.


M.C., and Wang, D. 2007. Factors impacting ethanol production from grain sorghum in
the dry-grind process. Cereal Chem. 84:130-136.

O’Brien, A. 2006b. Effects of amylase, corn protein, and corn fiber contents on production

Xie, X. J., and Seib, P. A. 2000. Laboratory procedure to wet-mill 100 g of grain sorghum into

Yang, P., and Seib, P. A. 1995. Low-input wet-milling of grain sorghum for readily accessible

lactic acid production as affected by sorghum genotype and location. Ind. Crops Prod.
18:245-255.

and the effect of protein. Cereal Chem. 75:710-713.
CHAPTER 2 - IMPACT OF MASHING ON SORGHUM PROTEINS AND ITS RELATIONSHIP TO ETHANOL FERMENTATION

ABSTRACT

Nine grain sorghum cultivars with a broad range of ethanol fermentation efficiencies were selected to characterize the changes in sorghum protein in digestibility, solubility, and microstructure during mashing and to relate those changes to ethanol fermentation quality of sorghum. Mashing reduced in vitro protein digestibility considerably and a large amount of polymers cross-linked by disulfide bonds were developed during mashing. As a marker of cross-linking, protein digestibility of the original samples was highly related to conversion efficiency. γ-kafirin (%) neither correlated to ethanol yield nor conversion efficiency significantly. Solubility of proteins in an alkaline borate buffer in conjunction with SDS decreased substantially after mashing. Solubility and the SE-HPLC area of proteins extracted from mashed samples were highly correlated with ethanol fermentation. Ethanol yield increased and conversion efficiency improved notably with the increase of extracted proteins from mashed samples. SE-HPLC total area could be used as an indicator to predict ethanol fermentation. CFLSM images proved that sorghum proteins tended to form highly extended, strong web-like microstructures during mashing. The degree of protein cross-linking differed among samples, and more open microstructures were observed in samples with higher conversion efficiencies. The web-like protein matrix was found to hold not only starch granules but also some oligosaccharides or polysaccharides inside. The formation of web-like microstructures due to cross-linking reduced conversion efficiency.

Keywords: Sorghum; protein; kafirin; digestibility; ethanol; fermentation; efficiency; HPLC; cross-linking; CFLSM; starch

INTRODUCTION

Interest in the production of fuel ethanol has increased significantly worldwide in recent years. World production reached an all-time high of nearly 13.5 billion gallons in 2006, and ethanol production in the U.S. is now undergoing unprecedented expansion with more than a 300% increase since 2000. According to the 2007 Renewable Fuels Association annual industry outlook (1), the U.S. ethanol industry produced a record 4.9 billion gallons of ethanol from 110 biorefineries located in 19 states across the country in 2006.

Sorghum (*Sorghum bicolor* L. Moench) is a drought-resistant and low-input cereal grain grown throughout the world, and interest in using it for bio-industrial applications is now growing (2). Although currently only about 2.5% of fuel ethanol is produced from grain sorghum, annual consumption of sorghum by the ethanol industry is steadily increasing from 11.3% in 2004, to 15% in 2005, and to 26% in 2006 (1, 3, 4).

Starch and protein are the two major components in sorghum grain. Recent research has shown that with sorghum, starch content is a good indicator of ethanol yield in the dry-grind process, but starch content itself could not explain conversion efficiency well (5).

Sorghum is rich in potential nitrogen for yeast growth during fermentation. However, a significant problem with sorghum is its comparably poor nutritional quality. Protein digestibility in wet-cooked sorghum is relatively lower compared with other cereals (6, 7), presumably through formation of strong protein cross-links that occur during cooking of the sorghum. This protein cross-linking may also reduce the availability of nitrogen in sorghum by the yeast preventing breakdown of sorghum proteins. Yeast cannot use complex nitrogenous materials for its growth unless the proteins are hydrolyzed to simple amino acids in terms of dipeptides or perhaps tripeptides (8).

Nevertheless, the effect of sorghum protein on ethanol fermentation is far beyond simple nutritional deficiencies. Among cereals, sorghum generally has the lowest starch digestibility because of the interactions between protein and starch, which may reduce the susceptibility of native and gelatinized starch to enzymatic hydrolysis (9). Sorghum grains with lower capacities for starch gelatinization were observed to have more kafirin-containing protein bodies, which may restrict the starch granules from fully gelatinizing, thereby resulting in lower digestibility (10). Neither the starch itself nor materials in the outer layers of sorghum seeds appeared to be related to poor starch digestibility, and treating flour with pepsin before cooking or cooking with
a reducing agent led to an increase in starch digestibility, suggesting that protein may act as a barrier to starch digestion (11).

Reduction in protein digestibility of cooked sorghum has generally been attributed to the formation of cross-linked protein polymers, which are resistant to proteolysis (7, 12-16). Duodu et al (15) found that protein digestibility decreased during kernel development and that this decrease paralleled the increase in disulfide-bonded proteins involving β- and γ-kafirins. It has been hypothesized that γ- and to a lesser extent β-kafirins form a disulfide-bound enzyme-resistant layer at the periphery of the protein bodies that restricts access by proteases to the more easily digestible α-kafirin (17). A scanning electron microscopy study (12) indicated that breakdown of sorghum storage proteins starts on the outside of the protein bodies and progresses toward the interior. Upon wet cooking, such as porridge making, an increase in disulfide cross-linked protein oligomers and polymers occurs (12, 14, 15). In vitro protein digestibility has thus become an important marker of protein cross-linking.

Several in vitro studies have shown that cooking sorghum with reducing agents improves its protein digestibility, supporting the role of disulfide cross-links on protein digestibility (7, 12, 14). However, use of sodium bisulfite as a reducing agent during cooking did not completely eliminate the problem of lowered sorghum protein digestibility on cooking (14). SDS-PAGE analyses displayed the formation of reduction-resistant oligomers (45-50 kDa) in cooked sorghum (15, 16). The observation that the digestibility was not fully reversed to the level of uncooked flour may be due to the presence of disulfide bonds inaccessible to the reducing agent, possibly due to the conformation of the proteins not allowing reducing agents easy access to disulfide bonds (14, 15). The possibility of formation of non-disulfide cross-links through oxidative coupling of tyrosine has also been suggested (18).

Among the five basic steps in the conventional dry grind ethanol process are grinding, cooking, liquefaction, saccharification, and fermentation. Mashing goes throughout the entire process beginning with mixing the grain meal with water (and possibly backset stillage) to delivery of a mash ready for fermentation. Mashing is a wet-cooking process that is expected to have similar effects on sorghum protein cross-linking as wet-cooking sorghum foods, such as porridge. The main difference between mashing and wet cooking is that during mashing, starch is converted into fermentable sugars, while wet cooking only gelatinizes starch granules. CFLSM images have shown that sorghum proteins tend to form highly extended, strong web-like
microstructures during mashing and small starch granules were firmly trapped within a web-like protein matrix (5). Thus, it appears that protein cross-linking during mashing of sorghum occurs in a similar fashion as when cooking sorghum for food production. This in turn means that the endosperm proteins of sorghum and their cross-linking may play an important role in determining ethanol yield and conversion efficiency by impairing the complete enzymatic digestion of sorghum starch to fermentable sugars. Therefore, the degree of cross-linking in sorghum proteins and the microstructure resulting from this cross-linking should be related to ethanol yield and conversion efficiency. The object of this study was to characterize the changes in sorghum proteins during mashing, examine the protein microstructure, and relate those changes to ethanol fermentation.

MATERIALS AND METHODS

Sample Preparation

Nine cultivars, from a 2004 commercial winter breeding nursery, were selected from a population of 70 proprietary sorghum genotypes and elite hybrids with a broad range of ethanol fermentation efficiencies. Of these nine cultivars, two contained tannins, while the remainders were tannin-free. Mashed samples were prepared according to procedures described by Wu et al (5) as follows: 30 g of original sample (dry matter) was liquefied by heat-stable α-amylase at 95 °C for 45 min and at 80 °C for 30 min, and was then saccharified by the addition of amylglucosidase at 60 °C for 30 min. After cooling to room temperature, all the mash in a 250-mL flask was collected and freeze-dried. Enzyme dosages were the same as those used in fermentation tests for ethanol production. Original samples for protein extraction and all mashed samples were ground using an Udy mill (Udy Corp., Fort Collins, CO) through a 0.25-mm screen.

Protein Digestibility Assay

In vitro protein digestibility tests were modified from the method of Mertz et al (19) as follows: 200 mg of unmashed samples or 280 mg of mashed samples were suspended in 35 mL or 49 mL of pepsin solution (1.5 g of enzyme/L of 0.1 M potassium phosphate buffer at pH 2.0) and incubated with vigorous shaking at 37 °C. Pepsin digestion was stopped at 2 h with the addition of 2 or 2.8 mL of 2 M NaOH, respectively. After centrifuging at 9050g for 15 min, the
supernatant was discarded and the residue was washed in 10 mL of 0.1 M phosphate buffer (pH 2.0) and centrifuged as before. After the second washing and centrifugation steps, the residue was frozen and then lyophilized. The freeze-dried residue was then weighed and analyzed for nitrogen content. The pepsin used was porcine pepsin 1:10000 (Sigma P-7000; activity 924 units per mg of protein). Cooked materials were prepared in two ways. One method (cooking1) was the procedure described by Hamaker et al (7) as follows: 200 mg of original samples were suspended in 2 mL of water in a 15-mL capped test tube and cooked at above 98°C for 20 min. Another method (cooking2) was similar to the mashing procedure in a fermentation test as follows: 200 mg of original samples were suspended in 2 mL of water in a 15-mL capped test tube and heated at 95°C for 45 min, 80°C for 30 min, and 60°C for 30 min. Cooked samples were then suspended in 35 mL of the pepsin solution, and their protein digestibilities were measured.

**Extraction of Kafirins**

Albumins and globulins were first extracted from the unmashed samples (100 mg) with 1 mL of 50 mM Tris-HCl at pH 7.8 containing 0.1 M KCl and 5 mM EDTA as described previously (20, 21). Samples were extracted in this manner twice for 5 min with continual vortexing. The pellets were then washed with 1 mL of water for 5 min. Finally, kafirins in the pellets were extracted with 1 mL of 60% (v/v) tert-butanol containing 0.5% (w/v) sodium acetate and 2% (v/v) β-ME using two 5- min extractions with the supernatant (centrifuged at 13200g for 4 min) from each extract pooled 1:1 to produce the final extract (22).

**Extraction of Proteins with Borate Buffer**

Samples (100 mg) were extracted for 24 h at room temperature with 12.5 mM sodium borate at pH 10.0 containing 2% (w/v) SDS (buffer 1) at a ratio of 1:10 (flour to solvent), using a VortexGenie2 equipped with a 30-place foam microfuge holder (Scientific instruments, Bohemia, NY). Samples were vortexed automatically at 5 min intervals during extraction. The suspension was centrifuged at 13200g for 4 min, and the supernatant was filtered through a syringe filter with a 0.45 µm membrane. The pellets were washed twice by buffer 1 and once by water, then lyophilized, weighed, and analyzed for nitrogen content. Soluble nitrogen was calculated by subtraction of nitrogen in a freeze-dried pellet from total nitrogen in unmashed or
mashed samples. Protein solubility was reported as the percentage of soluble nitrogen to total nitrogen.

**Extraction of Proteins with Borate Buffer plus β-ME**

A total of 100 mg of samples was extracted with 1 mL of 12.5 mM sodium borate at pH 10.0 containing 2% (w/v) SDS and 2% (v/v) β-ME (Buffer 2) for 30 min twice, with the supernatant from each extract pooled 1:1 to produce the final extract for RP-HPLC. During extraction, the samples were vortexed continuously.

**Sequential Extraction of Proteins with Borate Buffer and Borate Buffer plus β-ME**

A total of 100 mg of samples was first extracted with 1 mL of buffer 1 for 24 h. After centrifugation, the pellets were washed twice with buffer 1, and then extracted with 1 mL of buffer 2 for 30 min twice, with the supernatant from each extract pooled 1:1 to produce the final extract for RP-HPLC. The residues were then washed, lyophilized, weighed, and analyzed for nitrogen content in the same manner as the above extraction with buffer 1.

**Protein Characterization**

RP-HPLC separation of reduced proteins was conducted using an Agilent 1100 HPLC system equipped with a Jupitor C18 2.0×150 mm column (Phenomenex, Torrance, CA) with guard columns of the same material. A total of 10 μL of the samples was injected and separated with a continuous linear gradient of 0.1% TFA (solvent A) and ACN containing 0.1% TFA (solvent B), in which solvent B increased from 28 to 60.5% over 50 min and was then held 10 min. The flow rate was 0.5 mL/min, and column temperature was maintained at 50°C. SE-HPLC was conducted using an Agilent 1100 HPLC system with a 300×7.8 mm BioSep-SEC-S3000 column (Phenomenex, Torrance, CA). The mobile phase was a pH 7.0 sodium phosphate buffer (50 mM) with 1% SDS added. Column temperature was maintained at 40°C with a flow rate of 1 mL/min with a 15 μL injection volume. Standard proteins thyroglobulin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were analyzed to estimate the molecular-weight distribution of the sorghum proteins separated by SE-HPLC. All proteins were detected by measuring UV absorbance at 214 nm. Peak areas were expressed in arbitrary units, based on millivolts of detector output. In order to make data from different samples with different protein contents...
comparable, area per milligram of protein was calculated by dividing the integrated area by protein mass in a sample.

**CFLSM Images**

A total of 100 mg of mashed samples was first washed with 1 mL of H₂O 3 times. Proteins in the pellets were then labeled by mixing with 1 mL of 0.05% (w/v) FITC solution (in 0.5 mM NaOH) and incubating it in the dark for 1 h at room temperature. After centrifugation at 13200g for 4 min, the pellet was spread on a glass slide and allowed to dry at room temperature in the dark. Protein microstructure was visualized using a laser-scanning confocal microscope (Zeiss LSM 5 PASCAL, Carl Zeiss MicroImaging, Inc., Thornwood, NY). Prior to imaging, one drop of oil was added to the sample. A cover slip was placed on it, and another drop of oil was added on top of the cover slip to achieve higher resolution (23). Sorghum protein fluorescence was analyzed using 488 nm excitation and then through a 505-530 band-pass barrier filter for detection of FITC. Optical sections of samples were collected with a z step of 0.9 μm throughout the sample thickness. Three-dimensional images comprised greater than 25 laser-generated optical planes in z sectioning. Only one plane in the middle of the z series is presented.

**Glucose and Total Starch Assay**

Glucose and total starch content were determined using Megazyme total starch kits (24). For glucose analysis, 0.5 g of mashed samples was dispersed in 10 mL of H₂O. After the slurry was vortexed for 5 min, it was diluted to 250 mL, and glucose in the diluted solution was measured following the instructions of the manufacturer. For the residue starch analysis in mashed samples, about 0.5 g of samples were washed with 10 mL of 80% (v/v) ethanol or 10 mL of H₂O for six times to remove soluble sugars. In some experiments, proteins in mashed samples (0.5 g) were first extracted by different buffers (ratios of solvent to sample and extraction manners kept the same as those described in the protein extraction part). After that, the residual pellets were further washed with 10 mL of H₂O 3 times. DMSO was applied in all starch measurements. Results were reported as a percentage of the hydrolyzed starch existing only in glucose in mashed samples, or the residual starch in pellets to the total starch of their original samples.
Nitrogen Quantitation

Nitrogen content of original, mashed samples, and pellets after protein digestion was analyzed by combustion (25) using a Nitrogen Determinator (FP-528, Leco Corp., St. Joseph, MI). Nitrogen values were multiplied by 6.25 to convert to protein values.

Other Methods

Ethanol fermentation used the same procedure as described by Wu et al (26). Analysis of variance (ANOVA), least significant difference (LSD), and linear regression were performed using SAS software version 9.1 (27).

RESULTS

Impact of Mashing on Sorghum Protein Digestibility

As expected, in vitro protein digestibility decreased considerably after mashing (Table 2.1). Protein digestibility ranged from 23.0 to 68.2%, which was consistent with those data reported in the literature (15, 16, 19). Protein digestibility for these cultivars decreased to less than 26.0% after mashing. Decreases in protein digestibility because of cooking agreed with a recent study, which showed that the decrease in protein digestibility varied from 36 to 57% (16). Although there was a significant systematic difference between the two cooking methods (p<0.05), overall the protein digestibilities were highly correlated with each other (R²=0.99, linear regression using the data in Table 2.1).

Impact of Mashing on Sorghum Protein Solubility

As shown in Table 2.2, the majority of the total proteins were extracted from the unmashed samples with buffer 1 for 24 h with protein solubility ranging from 77.2 to 92.6% (86.4% in average). Protein solubility varied significantly among both the unmashed and mashed samples. Protein solubility decreased significantly after mashing.

To further characterize changes in sorghum proteins during mashing, a sequential extraction scheme was used where proteins were first extracted under non-reducing conditions using buffer 1 and then with reducing conditions using buffer 2. When the reducing buffer 2 was sequentially after buffer 1, additional protein was extracted in both the unmashed and mashed
samples. Mashed samples still showed lower overall protein solubility (37-84%) than did the unmashed (92-96%).

SE-HPLC Analysis of Proteins from Original and Mashed Sorghum

Typical SE-HPLC patterns of sorghum proteins extracted with buffer 1 (i.e. unreduced) are shown in Figure 2.1. For comparative purposes, chromatograms were divided into four regions (indicated as I, II, III, and IV in Figure 2.1). On the basis of comparisons to the elution times of standard proteins, fraction I is composed of proteins with $M_r$ greater than 669 kDa. For the unmashed samples, SE-HPLC curves were similar to those reported by other researchers (28, 29) with small amounts of large polymeric proteins (Peak I) but with the majority of the proteins eluting at around 8 min, where monomeric proteins are expected (28, 29). For the mashed samples, overall SE-HPLC peak areas were small, in agreement with Table 2.2. However, area ratios of peaks I and II were relatively large in the mashed samples.

RP-HPLC Analysis of Proteins from Original and Mashed Sorghum

Figure 2.2 shows the RP-HPLC separations of kafirins extracted from unmashed cultivars that vary in conversion efficiency. $\gamma$-kafirin (%) was calculated as a percentage of the area of $\gamma$-kafirin fraction to total area (including $\alpha$, $\beta$, and $\gamma$ fractions) in RP-HPLC chromatograms. $\gamma$-kafirin (%) ranged from 0.33 to 7.61% (3.97% in average), with the remainder of the kafirins being $\alpha$ and $\beta$. Figure 2.3 showed the RP-HPLC chromatograms of proteins extracted from some unmashed and mashed samples with buffer 2. According to previous works (22, 30), 90-95% of the total protein was expected to be extracted from the unmashed samples in Figure 2.3. However, less than 5% of the total protein was extracted from its mashed counterparts (calculated by the percentage of RP-HPLC total area). Fewer proteins were extracted from the cultivar with the lowest conversion efficiency with buffer 2, which contained tannins and thus more protein cross-linking would be expected because of protein–tannin interactions. Figure 2.4 further proved that most of the total protein in the unmashed samples had been extracted without using a reducing agent. Even if pre-extracted with buffer 1 for 24 h, more proteins had been extracted from mashed samples than those by direct extraction with buffer 2.
**Protein Microstructures**

CFLSM images confirmed that sorghum proteins tended to form highly extended, strong web-like microstructures during mashing (5). The cultivar with the lowest conversion efficiency formed a tightly cross-linked microstructure (Figure 2.5C), which could hold starch granules or polysaccharides inside or retard or prevent the access of enzymes to starch. Again, severe cross-linking in this sample was most likely because of a combination of heat-induced cross-linking and cross-linking because of protein–tannin interactions. More open web-like microstructures were observed in those cultivars with higher conversion efficiencies (Part A and B of Figure 5) upon mashing.

**Relationships between Protein Digestibility, Solubility, and Composition, and Ethanol Fermentation**

According to linear regression analyses on data in Table 2.1, it was found that protein digestibility of the unmashed sorghum was highly related to both ethanol yield ($R^2 = 0.567$, $p = 0.02$) and conversion efficiency ($R^2 = 0.515$, $p = 0.03$). The result of multiple linear regression showed that the role of protein digestibility was not significant ($p = 0.13$) when combined with starch to predict ethanol yield. Protein digestibility of the mashed sorghum was not correlated to fermentation parameters.

SE-HPLC total area per milligram of protein of proteins extracted with buffer 1 from the unmashed sorghum was highly related to conversion efficiency ($R^2 = 0.522$, $p = 0.03$), which is similar to the relationship between protein digestibility and efficiency. In the mashed samples, both protein solubility and SE-HPLC area were highly correlated with fermentation parameters (Table 2.3).

Due to its potential role in sorghum protein cross-linking, it was anticipated that γ-kafirin (%) would relate to ethanol fermentation, but it neither correlated to ethanol yield ($p = 0.18$) nor conversion efficiency ($p = 0.22$) significantly.

**Glucose and Total Starch Analysis in Mashed Samples**

Analyses of glucose found that only 49.6-61.0% of the total starch had been completely hydrolyzed to glucose in the mashed samples (Table 2.4), but such low levels of glucose before yeast inoculation did not affect final fermentation results because the enzyme, amyloglucosidase, was active throughout the fermentation process (i.e., simultaneous saccharification and
fermentation, SSF). Further sugar analyses on mashed samples by HPLC showed that maltose made up 13.9-24.1% of the total starch, while maltotriose accounted for 0.4-0.6% (data not shown). HPLC could not separate oligosaccharides and polysaccharides with glucose units greater than three, but a group of starch hydrolyzates with DP > 3 were present in the mashed samples and represented 17.0-24.9% of the total starch (calculated from the difference between total starch and sum of the starch which had been hydrolyzed to glucose, maltose, and maltotriose). Following the standard procedure for total starch assay of samples containing glucose and malthosaccharides (24), mashed samples were first washed with 80% aqueous ethanol and 9.5-17.9% of the total starch was measured as residual starch. However, the residual starch decreased to only 1.3-3.7% of the total starch when mashed samples were washed with water instead of aqueous ethanol. It is obvious that some oligosaccharides or polysaccharides were soluble in water but insoluble in 80% ethanol and that they accounted for 7.9-15.2% of the total starch in the mashed samples (calculated by the difference between values in the third and fourth columns in Table 2.4).

DISCUSSION

In order to investigate the role of protein cross-linking in sorghum, several different methods were employed to provide insight into the amount of cross-linking occurring during the mashing process with sorghum samples varying in conversion efficiencies. Two sorghum samples with tannins were included in this set to extend the range of ethanol efficiencies and to provide extremes in protein cross-linking. In these particular samples, protein cross-linking would be due to both heat-induced cross-links as well as to protein–tannin interactions.

The first such indicator employed was protein digestibility as measured using a pepsin assay. Protein cross-linking is known to reduce digestibility in sorghum proteins, thus by evaluating the protein digestibility in uncooked and mashed sorghum samples, the degree of cross-linking could be determined indirectly. Two cooking methods were used to compare the effects of mashing process on protein digestibility. Generally, mashing led to a greater decrease in protein digestibility than both cooking methods tested (Table 2.1). Protein digestibility of the original samples may serve as a marker for protein cross-linking related to ethanol production. It is certain that most of the proteins in mashed samples would not be directly digested by yeast, especially for those with high Mr values, such as fraction I in Figure 2.1, because the
fermentation environment was much milder than pepsin digestion and buffer extraction conditions. The role of protein solubility to predict ethanol fermentation could be related to protein structures, which can determine the access of enzymes to sorghum starch.

Although protein digestibility was found to be related to ethanol production, the pepsin assay did not mimic conditions used in the mashing of sorghum. Therefore, additional studies were conducted on protein solubility. Protein solubility should decrease when the proteins cross-link during mashing, and thus, protein solubility would serve as another indicator for the degree of cross-linking occurring during mashing. For the unmashed sorghum, both extraction procedures extracted the majority of the protein. Differences between the first and second columns in Table 2.2 would reflect the amount of additional proteins extracted using a reducing agent. Those would presumably be the largest, most-difficult-to-remove polymeric proteins from sorghum, while the smaller and easier-to-extract polymeric proteins would be extracted with buffer 1 (similar in nature to the soluble polymeric and insoluble polymeric proteins of wheat). When looking at the data for the mashed sorghum in Table 2.2, the effect of mashing on sorghum proteins can be clearly seen. For original sorghum, buffer 1 extracted 77-93% of total protein. After mashing, protein solubility dropped to only 8-31%. When the sequential extraction scheme was applied, protein solubility was still only 37-84%. Thus, mashing caused large decreases in protein solubility that were only partially recovered when a reducing agent was used in the extraction process. One possible explain for this is that the disulfide-mediated polymerization of kafirins upon mashing was so extensive that it retarded disulphide bonds accessible to β-ME or kafirins to pepsin, and resulted in low protein solubility and digestibility. With the removal of some aggregating proteins by pre-extraction with buffer 1 for 24 h, the structure of the cross-linked web-like protein matrix could have been loosened or weakened. RP-HPLC separations (Figures 2.3 and 2.4) demonstrated that pre-extraction with buffer 1 did help β-ME to extract more polymers which were cross-linked by disulfide bonds than direct extract with β-ME. However, there might be non-disulphide cross-links existing in sorghum proteins after mashing (18).

SE-HPLC results mirrored the protein solubility data; i.e., less protein was seen in the SE-HPLC chromatograms from the mashed samples than in the original samples. As can be seen in Table 2.3, the more proteins extracted with buffer 1 or sequentially with buffer 2, the higher the ethanol yield and conversion efficiency of fermentation. If changes in protein solubility are
taken as related to changes in protein cross-linking, then the more protein cross-links during mashing, the poorer the fermentation performance. Likewise, the amount of total area in the SE-HPLC chromatograms was slightly better correlated to fermentation parameters than protein solubility, as measured by nitrogen combustion. There was a strongly linear correlation between SE-HPLC total area per milligram of protein and ethanol yield, as well as conversion efficiency (Table 2.3). The result of multiple linear regression showed that the role of SE-HPLC total area was dominant (p = 0.016), even when combined with starch to predict ethanol yield. SE-HPLC total area could be used as an indicator to predict ethanol fermentation. Again, the area under the SE-HPLC chromatograms would be expected to be related to protein cross-linking – the more cross-linked the samples, the less material available for SE-HPLC analysis. The peak area for the largest polymeric peak, fraction I, was also highly correlated to both ethanol yield and conversion efficiency.

Attempts to investigate whether individual subunits may play a role in governing ethanol fermentation from sorghum did not reveal any correlations to α-, β-, or γ-kafirins. Cross-linking occurring during mashing is most likely a complex process with multiple factors responsible (31).

The above results indicate that protein cross-linking does play a role in the production of ethanol from sorghum, albeit through indirect measures of protein cross-linking. Thus, CFLSM was employed to provide further understanding of the role of such cross-linking in the process.

CFLSM images displayed some small starch granules which were firmly trapped within a web-like protein matrix (5), but un-gelatinized starch granules were not often to be viewed for all mashed samples especially those with high conversion efficiency. There was about 5-6% of total starch in sorghum distillers dried grains with solubles, DDGS (32), indicating those starch granules, oligosaccharides, or polysaccharides holding tightly by protein matrix would not be utilized by yeast fermentation. In conclusion, highly cross-linked protein matrixes may impair complete enzymatic digestion of sorghum starch to fermentable sugars by holding starch granules, oligosaccharides, or polysaccharides inside or retarding or preventing enzyme accessibility to gelatinized starch.

Results of total starch assay on residual pellets after protein extraction demonstrated that the web-like matrix held not only starch granules but also some oligosaccharides or polysaccharides inside. As shown in Table 2.4, some water-soluble oligosaccharides or
polysaccharides were released gradually following multiple steps of protein extractions because of weakening or partial rupture of the protein cross-linking. Thus, the protein cross-linking of sorghum proteins does appear to trap starch and make it less available to enzymes during the fermentation process.

ABBREVIATIONS USED

CAN, acetonitrile; β-ME (2-ME), beta-mercaptoethanol; CFLSM, confocal laser-scanning microscopy; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; SE-HPLC, size-exclusion high-performance liquid chromatography; TFA, tri-fluoroacetic acid; Tris-HCl, 2-Amino-2-(hydroxymethyl)-1, 3-propanediol, hydrochloride.

ACKNOWLEDGEMENT

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LITERATURE CITED


Figure 2.1 Typical SE-HPLC separations of proteins extracted with buffer 1 (12.5 mM sodium borate at pH 10.0 containing 2% SDS) from original and mashed sorghum samples with different conversion efficiencies.
Figure 2.2 RP-HPLC separations of kafirins extracted from unmashed sorghum samples with different conversion efficiencies.
Figure 2.3 RP-HPLC separations of proteins extracted with buffer 2 (12.5 mM sodium borate at pH 10.0 containing 2% SDS and 2% β-ME) for 30 min twice from unmashed and mashed sorghum samples with different conversion efficiencies.
Figure 2.4 RP-HPLC separations of proteins extracted with buffer 2 (12.5 mM sodium borate at pH 10.0 containing 2% SDS and 2% β-ME) after extraction with buffer 1 (12.5 mM sodium borate at pH 10.0 containing 2% SDS) for 24 h from unmashed and mashed sorghum samples with different conversion efficiencies.
Figure 2.5 CFSLM images (single optical planes) of mashed sorghum samples with different conversion efficiencies, protein matrix (green areas) stained with FITC.
### Table 2.1 In Vitro Protein Digestibility and Fermentation Parameters

<table>
<thead>
<tr>
<th>Variety Code</th>
<th>Protein Digestibility (%)</th>
<th>Fermentation Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*original&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>mashing&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>I</td>
<td>54.1 &lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.7 &lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>II</td>
<td>48.4 &lt;sup&gt;e&lt;/sup&gt;</td>
<td>18.3 &lt;sup&gt;c, d&lt;/sup&gt;</td>
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<tr>
<td>III</td>
<td>23.0 &lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.6 &lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>IV</td>
<td>64.1 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.0 &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V</td>
<td>33.6 &lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.0 &lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>VI</td>
<td>63.9 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.4 &lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>VII</td>
<td>58.4 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.1 &lt;sup&gt;c&lt;/sup&gt;</td>
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<td>19.5 &lt;sup&gt;c&lt;/sup&gt;</td>
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<td>25.3 &lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Standard Error</td>
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<td>LSD (0.05)</td>
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<td>2.08</td>
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</table>

<sup>A</sup> Capital in superscript in the second row mean significantly different (p<0.05) among the treatments. <sup>B</sup> Cooking 1 means the method described by Hamaker et al (7), and cooking 2 means the method similar to the mashing procedure without enzymes added. <sup>C</sup> Values followed by the same letter in the same column are not significantly different (p<0.05).
Table 2.2 Protein Solubility in Borate Buffer with or without Reducing Agent Added

<table>
<thead>
<tr>
<th>variety code</th>
<th>protein solubility (%)</th>
<th>original sorghum</th>
<th>mashed sorghum</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>extraction with buffer 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>sequential extraction with buffer 1 and buffer 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>extraction with buffer 1</td>
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<td>I</td>
<td>83.9 d&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95.1 a</td>
<td>19.8 d</td>
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<td>II</td>
<td>89.1 b</td>
<td>95.4 a</td>
<td>20.3 d</td>
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<tr>
<td>III</td>
<td>77.2 e</td>
<td>93.3 b</td>
<td>8.3 f</td>
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<td>IV</td>
<td>92.3 a</td>
<td>95.8 a</td>
<td>31.1 a</td>
</tr>
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<td>V</td>
<td>81.5 e</td>
<td>93.6 b</td>
<td>7.8 f</td>
</tr>
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<td>VI</td>
<td>85.6 c</td>
<td>93.2 b</td>
<td>16.7 e</td>
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<td>95.2 a</td>
<td>27.6 b, c</td>
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<td>VIII</td>
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<td>92.5 b, c</td>
<td>27.1 c</td>
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<tr>
<td>IX</td>
<td>89.6 b</td>
<td>92.0 c</td>
<td>30.0 a, b</td>
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</table>

Replications 3 3 3 3
Standard error 0.52 0.41 0.92 0.71
LSD (0.05) 1.54 1.21 2.73 2.12

<sup>a</sup> Buffer 1 is 12.5 mM sodium borate at pH 10.0 containing 2% SDS, and buffer 2 is 12.5 mM sodium borate at pH 10.0 containing 2% SDS and 2% β-ME. <sup>b</sup> Values followed by the same letter in the same column are not significantly different (p<0.05).
<table>
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<th>ethanol fermentation</th>
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<td>0.597$^b$</td>
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<tr>
<td>conversion efficiency</td>
<td>0.545$^b$</td>
<td>0.532$^b$</td>
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$^a$ Buffer 1 is 12.5 mM sodium borate at pH 10.0 containing 2% SDS, and buffer 2 is 12.5 mM sodium borate at pH 10.0 containing 2% SDS and 2% β-ME. $^b$ Significant at 5% level. $^c$ Significant at 1% level. $^d$ Significant at 0.1% level.
<table>
<thead>
<tr>
<th>variety code</th>
<th>glucose in mashed sorghum (%)</th>
<th>total starch in various residues (%)</th>
<th>mashed sample</th>
<th>washing with 80% ethanol</th>
<th>washing with water&lt;sup&gt;Ab&lt;/sup&gt;</th>
<th>extraction with buffer 1&lt;sup&gt;B&lt;/sup&gt;</th>
<th>sequential extraction with buffer 1 and buffer 2&lt;sup&gt;C&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>55.4 e&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>12.0 f</td>
<td>2.6 e</td>
<td>1.3 e</td>
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<td></td>
<td>10.0 g</td>
<td>1.5 h</td>
<td>0.7 g</td>
<td>0.4 g</td>
</tr>
<tr>
<td>VIII</td>
<td>60.4 a, b</td>
<td></td>
<td></td>
<td>13.8 e</td>
<td>3.2 b</td>
<td>1.7 b</td>
<td>1.2 d</td>
</tr>
<tr>
<td>IX</td>
<td>49.6 g</td>
<td></td>
<td></td>
<td>16.5 c</td>
<td>1.3 i</td>
<td>0.3 h</td>
<td>0.2 h</td>
</tr>
</tbody>
</table>

replications: 2 2 2 2 2 2 2

standard error: 0.44 0.08 0.02 0.01 0.01 0.02 0.02

LSD (0.05) 1.40 0.27 0.06 0.03 0.02

<sup>a</sup> All values were calculated as a percentage of glucose or residual starch content to total starch content. <sup>b</sup> Capitals in superscript in the third row mean significantly different (p<0.05) among the treatments. <sup>c</sup> Values followed by the same letter in the same column are not significantly different (p<0.05).
CHAPTER 3 - SORGHUM PROTEIN EXTRACTION BY SONICATION AND ITS RELATIONSHIP TO ETHANOL FERMENTATION

ABSTRACT

The objectives of this research were to develop a rapid method for extracting proteins from mashed and nonmashed sorghum meal using sonication (ultrasound), and to determine the relationships between the levels of extractable proteins and ethanol fermentation properties. Nine grain sorghum hybrids with a broad range of ethanol fermentation efficiencies were used. Proteins were extracted in an alkaline borate buffer using sonication and characterized and quantified by size-exclusion HPLC. A 30-sec sonication treatment extracted a lower level of proteins from nonmashed sorghum meal than extracting the proteins for 24 hr with buffer only (no sonication). However, more protein was extracted by sonication from the mashed samples than from the buffer-only 24-hr extraction. In addition, sonication extracted more polymeric proteins from both the mashed and nonmashed samples compared with the buffer-only extraction method. Confocal laser-scanning microscopy images showed that the web-like protein microstructures were disrupted during sonication. The results showed that there were strong relationships between extractable proteins and fermentation parameters. Ethanol yield increased and conversion efficiency improved significantly as the amount of extractable proteins from sonication of mashed samples increased. The absolute amounts of polymeric proteins extracted through sonication were also highly related to ethanol fermentation. Thus, the SE-HPLC area of proteins extracted from mashed sorghum using sonication could be used as an indicator for predicting fermentation quality of sorghum.

Keywords: Sorghum; protein; polymer; sonication; solubility; ethanol; fermentation; efficiency; HPLC; cross-linking; CFLSM

2 Results have been published. R. Zhao, S. Bean and D. Wang. 2008. Sorghum protein extraction by sonication and its relationship to ethanol fermentation. Cereal Chem. 85: 837-842
INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) is a drought-resistant and low-input cereal grain grown throughout the world and the interest in using it for bioindustrial applications is now growing in the United States (Farrell et al 2006). Although currently only ≈2.5% of fuel ethanol is produced from grain sorghum, annual consumption of sorghum by the ethanol industry is steadily increasing from 11.25% in 2004 to 15% in 2005, and to 26% in 2006 (Renewable Fuels Association 2005, 2006, 2007). Researchers and ethanol producers have shown that grain sorghum is a viable feedstock (technically acceptable, fits the infrastructure, and can be economically viable) for ethanol, and could make a larger contribution to the nation’s fuel ethanol requirements.

Starch and protein are the two major components in sorghum grain. Recent research has shown that starch content is a good indicator of ethanol yield in the dry-grind process, but starch content itself could not explain conversion efficiency well (Wu et al 2007). Sorghum varies in protein content from 6 to 18%, with 70-90% of the total protein belonging to the storage proteins (kafirins) (Lookhart et al 2000). According to previous research with 68 sorghum cultivars, a strong negative correlation was observed between ethanol yield and protein content (*R*² = 0.60, *p* < 0.01) (unpublished data), which is similar to data reported for soft wheat varieties (Swanstion et al 2007). However, multiple linear regression, including both starch and protein content as predictors, verified that protein content did not significantly contribute to ethanol yield (*p* = 0.395). The effect of protein content on conversion efficiency was statistically significant (*p* = 0.015), but represented only 8.6% of variation in efficiency (unpublished data).

Recently, we investigated the role of protein cross-linking to determine its impact on ethanol production. Protein digestibility, solubility and microstructures were characterized for insight into protein cross-linking occurring during the mashing process (Zhao et al 2008). Protein digestibility decreased significantly during mashing to levels lower than found in cooked sorghum foods. Likewise, protein solubility in SDS buffer with alkaline borate decreased substantially after mashing. Confocal laser-scanning microscopy (CFLSM) images showed that web-like protein cross-links formed during mashing could trap oligosaccharides, polysaccharides, or starch and reduce availability to enzymes during ethanol production (Wu et al 2007; Zhao et al 2008).
Therefore, protein cross-linking does have a significant effect on production of ethanol from sorghum (Zhao et al. 2008). Protein digestibility, used as a marker of protein cross-linking of nonmashed sorghum meal, and protein solubility parameters showed positive correlation with conversion efficiency of sorghum. It is certain that most of the proteins soluble in the borate buffer would not be digested by yeast directly (Berry and Brown 1987). The role of protein solubility to predict ethanol fermentation could be related to protein structures which can determine the access of enzymes to native and gelatinized starch, polysaccharides, or oligosaccharides (Rooney and Pflugfelder 1986; Zhao et al. 2008). The amount of total area under SE-HPLC had a better correlation with fermentation parameters than protein solubility and could be used as an indicator to predict ethanol-production quality of sorghum (Zhao et al. 2008).

It is promising that the two indicators of degree of cross-linking occurring during mashing, protein digestibility and solubility, were highly correlated to fermentation parameters. To date, there is no rapid method that can be used to predict conversion efficiency of sorghum except for direct laboratory fermentation procedures. SE-HPLC has been widely used and provides automatic analysis, high accuracy, and utilizes only a small amount of sample. Therefore, SE-HPLC could be considered as a good system for rapid characterization and quantization of proteins extracted from sorghum for predicting ethanol fermentation parameters. Sonication was used as a rapid method for extraction of unreduced wheat flour proteins by breaking down the glutenin fraction with the largest molecular size (Singh et al 1990; Morel et al 2000; Singh and MacRitchie 2001) presumably by the mechanical shear degradation (MacRitchie 1975; Singh et al 1990). Ultrasound has also been used to enhance the extraction of sorghum proteins (Bean et al 2006; EI Nour et al 1998). A method for isolating sorghum starch was also developed using sonication combined with buffers (Park et al 2006), that proved the effectiveness of sonication to separate starch from protein matrix rapidly.

A quick method having a similar function as the 24-hr SDS borate buffer extraction method for sorghum protein extraction (Zhao et al. 2008) is needed for prediction of ethanol fermentation. Therefore, the combination of sonication with the extraction buffer and separation of proteins by SE-HPLC would make the extraction and analysis more efficient. Thus, the objective of this study was to develop a rapid method for sorghum protein extraction and analysis to predict ethanol fermentation.
MATERIALS AND METHODS

Sample Preparation

The nine sorghum hybrids (I–IX) described by Zhao et al (2008) were used and the methods for preparation of both mashed and nonmashed samples were unchanged in this study.

Extraction of Proteins Using Sonication

In total, 100 mg of nonmashed or mashed sorghum meal samples was mixed with 1 mL of 12.5 mM sodium borate, pH 10.0, containing 2% (w/v) SDS by vortexing for 10 sec in a 2-mL centrifuge tube to disperse the flour. The slurry was then sonicated with a Fisher F60 sonic dismembrator at an output of 10W for 30 sec. To prevent heat buildup, sample tubes were placed in an ice bath during sonication. After centrifugation at 13,200 × g for 4 min, the supernatant was filtered through a 0.45-μm membrane and analyzed directly by SE-HPLC. A separate 300 μL of the filtrate was transferred by a pipette to a 2-mL vial and mixed with 6 μL of β-mercaptoethanol (β-ME) to analyze the proteins under reducing conditions. For mashed samples, pellets were washed twice with the above borate buffer and once by water and then lyophilized, weighed, and analyzed for nitrogen content. Soluble nitrogen was calculated by subtraction of nitrogen in a freeze-dried pellet from total nitrogen in a mashed sample. Protein solubility was reported as the percentage of soluble nitrogen to total nitrogen.

Sequential Extraction of Proteins Using Sonication and Reducing Agent

In total, 100 mg of samples was first extracted using sonication as above. After centrifugation, the pellets were washed twice with 12.5 mM sodium borate, pH 10.0, containing 2% (w/v) SDS and then extracted twice for 30 min for each extraction with 1 mL of 12.5 mM sodium borate, pH 10.0, containing 2% (w/v) SDS and 2% (v/v) β-ME. The supernatant from each extract was pooled 1:1 to produce the final extract for RP-HPLC.

Protein Characterization

SE-HPLC separation of extracted proteins was conducted using an Agilent 1100 HPLC system with a 300 × 7.8 mm BioSep-SEC-S3000 column (Phenomenex, Torrance, CA). The mobile phase was a pH 7.0 sodium phosphate buffer (50 mM) with 1% SDS (w/v) added. Flow rate was of 1 mL/min with 15 μL injection volume and column temperature was maintained at
40°C. Standard proteins including thyroglobulin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were used to estimate $M_w$ distribution of sorghum proteins separated by SE-HPLC. RP-HPLC separation of reduced proteins was conducted using an Agilent 1100 HPLC system equipped with a Jupiter C18 2.0 × 150 mm column (Phenomenex) with guard columns of the same material. Samples (10 μL each) were injected and separated with a continuous linear gradient of 0·1% tri-fluoroacetic (TFA) (solvent A) and acetonitrile containing 0·1% TFA (solvent B), in which solvent B increased from 28 to 60.5% over 50 min and then was held 10 min (Bean et al 2000). Flow rate was 0.5 mL/min with column temperature maintained at 50°C. Proteins were detected by measuring UV absorbance at 214 nm. Peak areas were expressed in arbitrary units, based on millivolts of detector output.

**CFLSM Images**

Mashed sample (100 mg) was first washed with 1 mL of H₂O three times. Proteins in a residual pellet were labeled by mixing with 1 mL of 0.05% (w/v) fluorescein isothiocyanate (FITC) solution (in 0.5 mM NaOH) and incubating it in the dark for 1 hr at room temperature. After centrifugation at 13,200 × g for 4 min, the pellet was spread on a glass slide and allowed to dry at room temperature in the dark. Protein microstructure was visualized using a laser-scanning confocal microscope (Zeiss LSM 5 PASCAL; Carl Zeiss MicroImaging, Inc., Thornwood, NY). Before imaging, one drop of optical liquid immersion oil was added to the sample. A coverslip was placed on it, and another drop of oil was added on top of the coverslip to achieve higher resolution (Lee et al 2001). Sorghum protein fluorescence was analyzed using 488-nm excitation then through a 505-530 band-pass barrier filter for detection of FITC. Optical sections of samples were collected with a z-step of 0.9 μm throughout the sample thickness. Three-dimensional images included >25 laser-generated optical planes in z-sectioning. Only one plane in the middle of the z-series is presented.

**Nitrogen Quantitation**

Nitrogen content of nonmashed and mashed samples and pellets after sonication extraction was analyzed by combustion according to Approved Method 46-30 (AACC International 2000) using a nitrogen determinator (Model FP-528, Leco Corp., St. Joseph, MI). Nitrogen values were multiplied by 6.25 to convert to protein values.
Statistical Analysis

All experiments were performed at least in duplicate. Results presented are the mean values of the repeated experiments. Analysis of variance, least significant difference, and linear regression were performed using SAS software (v.9.1, SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Sonication Extraction and Protein Solubility

By using the method of Wallace et al (1990), 91.3-95.7% of the total protein in whole sorghum grain was extracted using 12.5 mM borate buffer, pH 10.0, containing 1% SDS and 2% β-ME with extraction time for 1 hr (Hamaker et al 1995). When the detergent for extracting sorghum proteins was optimized to be 2% SDS at pH 10.0 with a 35-80% extraction time reduction, the amount of extracted protein was similar compared to the control methodology (Park and Bean 2003). For mashed sorghum, <5% of the total protein could be extracted using the above optimum buffer with two 30-min extractions under reducing conditions (Zhao et al 2008). Without using reducing agent, 77.2-92.6% of the total protein was extracted from nonmashed sorghum with the borate buffer when extracted for 24 hr, but only 7.8-31.1% of the total protein was extracted from mashed sorghum (Zhao et al 2008). To increase the extraction rate, 12.5 mM sodium borate, pH 10.0, containing 2% SDS was used as extraction solvent in this study.

Previous research indicated that the power setting and sonication time are the two main variables affecting protein extractability and size distribution when using ultrasound (Singh et al 1990; Morel et al 2000; Singh and MacRitchie 2001; Bean et al 2006). In other words, protein extraction rate is a function of ultrasonic energy (sonication time × power) (Morel et al 2000). For example, most of proteins from strong as well as weak wheat flours can be extracted using a 30-sec sonication at power setting 5 (an output of 10W) (Singh et al 1990; Singh and MacRitchie 2001), and the solid-to-solvent ratio of 1:10–1:90 did not significantly affect the extractability when enough ultrasonic energy delivered to the flour sample (Singh et al 1990). However, oversonation could cause excessive depolymerization of glutenin (Singh et al 1990; Morel et al 2000). Our research showed that the optimum combination of power output and sonication time...
for extracting sorghum proteins is 10W for 30 sec, in which the maximum level of proteins can be extracted from original sorghum without oversonication (unpublished data).

Both solubility and SE-HPLC peak area of proteins extracted from mashed sorghum using the borate buffer with extraction time of 24 hr were highly correlated to ethanol fermentation (Zhao et al 2008). Ethanol yield increased and conversion efficiency improved notably with the increase of extracted proteins from mashed sorghum. After the encouraging results, we reproduced these effects using sonication in conjunction with the SDS borate buffer. By comparison with total area under the SE-HPLC chromatograms, the 30-sec sonication extracted fewer proteins from nonmashed sorghum than the 24-hr extraction with SDS borate buffer. The total area of proteins extracted using sonication accounted for 68.4-93.7% of the total area of those proteins extracted with the SDS borate buffer using a 24-hr extraction period (79.2% on average) (data not shown). Given that 77.2-92.6% of the total protein was extracted from nonmashed sorghum with the SDS borate buffer and a 24-hr extraction (Zhao et al 2008), the 30-sec sonication could extract 52.8-86.5% of the total protein from the nonmashed sorghum (calculated by product of protein solubility with SDS borate buffer and the percentage of total area by sonication to that by the SDS borate buffer).

SE-HPLC total peak area from sonication extraction decreased substantially after mashing (p < 0.0001) (Figure 3.1). Fewer proteins were extracted from mashed samples by sonication than their original counterparts, which coincides with the results that mashing reduced protein digestibility and solubility due to protein cross-linking (Zhao et al 2008). There was no significant difference in SE-HPLC total area between proteins extracted with the SDS borate buffer for 24 hr and sonication extraction from mashed samples (p = 0.08). Protein solubilities using sonication were 12.0-31.1% with an average of 24.0%, slightly higher than those using the 24-hr SDS borate buffer extraction (p < 0.0001) (data not shown).

**Protein Characterization by SE-HPLC**

Typical SE-HPLC patterns of proteins extracted using sonication are shown in Figure 3.2. For comparative purposes, the corresponding chromatograms of proteins using the 24-hr SDS borate buffer extraction in the previous study (Zhao et al 2008) are also displayed in Figures 3.2 and 3.3. SE-HPLC chromatograms were divided into four regions (fraction I, II, III, and IV) (Zhao et al 2008). Based on the elution times of standard proteins, fraction I was considered as
proteins with $M_w > 669$ kDa. It is noteworthy that all nonmashed sorghum samples had fraction I, with $M_w$ much larger than individual kafirins ($\approx 20$-30 kDa). This result confirmed the fact that a few large polymeric proteins already existed in nonmashed sorghum and were solubilized in some solvents with or without sonication (El Nour et al 1998; Bean et al 2006; Zhao et al 2008).

For nonmashed sorghum, the proteins extracted with the borate buffer had larger total area than those extracted using sonication. Sonication tended to extract more polymeric proteins than borate buffer, which is clearly evident from Figures 3.2 and 3.3. The chromatogram using sonication had bigger fraction I area as well as larger area ratio of fraction II. The chromatogram of proteins extracted with borate buffer was much sharper, with a majority of the proteins eluting at $\approx 8$ min. In contrast, the chromatogram obtained using sonication appeared flattened with diffused peaks, and more proteins were eluted before 8 min. This flattening trend became more obvious for samples with lower conversion efficiency in Figure 3.3. The increase in protein components eluted in fraction I and II using sonication extraction could be ascribed to the shear degradation of polymeric proteins that cannot be achieved by using the borate buffer alone. Sonication is believed to reduce the molecular weight of protein complexes by breaking covalent bonds, thus rendering them soluble (MacRitchie 1975; Singh et al 1990). Presumably, sonication extracted more polymeric proteins from the nonmashed samples with poorer conversion efficiency due to higher degree of protein cross-linking in those samples, as indicated by the lower protein digestibility in the previous study (Zhao et al 2008). It should also be pointed out that insolubility of proteins could result from factors other than simply $M_w$. For example, the proteins not easily extracted in buffer alone could be strongly aggregated through hydrophobic or other bonds. Changes to protein structure such as increases in $\beta$ sheet may impede access of solvent to aggregated proteins (Byaruhanga et al 2006), reducing their solubility.

For mashed sorghum, the difference in SE-HPLC total area of proteins extracted by both methods was not significant, as mentioned above. The proteins extracted through sonication had more peak area for fraction I than those extracted with the SDS borate buffer alone, while the latter had higher proportions of fraction II and III. The chromatogram from mashed sorghum extracted using sonication slightly skewed left in comparison with the other three curves, indicating a broader $M_w$ range (Figure 3.2). The protein solubility of the mashed sample with low conversion efficiency in Figure 3.3 was only 12.0%, which was much less than its counterparts (30.1 and 22.8% for the samples with high and medium conversion efficiency, respectively).
Moreover, the $M_w$ range of the proteins from this mashed sample became narrower in comparison with the other two samples with higher efficiency in Figure 3.3. The variation in protein extractability of mashed sorghum under sonication corresponds to the differences in the ease of degradation of different polymers (Singh and MacRitchie 2001).

The 30-sec sonication extracted more cross-linked polymeric proteins from mashed sorghum than the 24-hr SDS borate buffer extraction. However, because only 12.0-31.1% of the total protein in mashed sorghum was soluble with sonication extraction, fraction I accounted for only 1.7-6.3% of the total protein (calculated by product of solubility and area ratio of fraction I).

For proteins extracted from mashed sorghum using sonication and separated by SE-HPLC (Figure 3.4), the area of fraction I decreased significantly after reduction by 2% β-ME. It is obvious that high $M_w$ polypeptides decreased, while low $M_w$ polypeptides increased, thus indicating some of the proteins were linked by disulphide bonds. This is consistent with a recent report in which the polymeric proteins extracted from nonmashed sorghum and analyzed under nonreducing conditions by SE-HPLC were found to be disulfide linked together (Bean et al 2006) and have been reported in studies on cooked sorghum foods (Hamaker et al 1986, 1987; Duodu et al 2002; Ezeogu et al 2005). However, fraction I did not disappear completely after adding β-ME, indicating that some proteins could not be degraded by this reducing agent. Those protein molecules that still appeared at fraction I were taken as non-disulphide cross-linked polymers or, at the minimum, resistant to reducing agents.

**Sequential Extraction Using Sonication and Reducing Agent, and RP-HPLC Measurement**

Borate buffer (12.5 mM, pH 10.0) containing 2% SDS and 2% β-ME was used to extract the residual protein in pellets after sonication. It was expected that 90-95% of the total protein in nonmashed sorghum could be dissolved in this solvent with two extractions of 30 min each (Hamaker et al 1995; Park and Bean 2003; Nunes et al 2004). RP-HPLC chromatograms in Figure 3.5 show that the 30-sec sonication failed to dissolve all proteins in nonmashed sorghum and more proteins were further extracted under reducing conditions. Figure 3.5 also confirms that sonication preextracted more proteins from nonmashed sorghum with higher efficiency than those with lower efficiency and thus fewer proteins were extracted with β-ME and detected by RP-HPLC.
In the previous study (Zhao et al. 2008), β-ME further extracted 29.0-53.9% (44.0% on average) of the total protein from mashed residual sorghum pellets after preextraction with the SDS borate buffer for 24 hr, which demonstrated that a large amount of polymers cross-linked by disulfide bonds developed during mashing. There was not much difference in RP-HPLC patterns between proteins extracted by β-ME from residual pellets either after sonication or after pre-extraction with borate buffer for 24 hr. However, the total area under an RP-HPLC chromatogram of proteins extracted after sonication was lower than extracted after the 24-hr SDS buffer extraction for all mashed samples (data not shown). This indicates that fewer proteins were extracted by β-ME from the residual pellets after preextraction by sonication. Although the solubility of proteins extracted using sonication was slightly higher than that extracted by the SDS borate buffer for 24 hr, it could not compensate for the difference in the area of RP-HPLC chromatograms. It is likely that the cross-linking in sections of protein matrix broken by sonication was intact enough to reduce access to remaining disulphide bonds by reducing agents. Zhao et al. (2008) found that preextraction with the SDS borate buffer for 24 hr did help β-ME to extract more polymers cross-linked by disulfide bonds than direct extraction with β-ME.

Even sequential extraction using sonication or with the borate buffer, and β-ME could not completely solubilize proteins in mashed sorghum. This result contrasts with a recent report in which the protein in cooked sorghum was completely dissolved in solvent with 12.5 mM borate (pH 10.0), 2% (w/v) SDS, and 1% (v/v) β-ME (Nunes et al. 2004).

**CFLSM Images and Protein Microstructures**

CFLSM images proved that sorghum proteins tended to form highly extended, strong, web-like microstructures during mashing (Wu et al. 2007; Zhao et al. 2008). More open microstructures were observed in hybrids with higher conversion efficiencies (Zhao et al. 2008). The changes of protein microstructure in a mashed sample during sequential extraction are shown in Figure 3.6. The sample selected had the most obvious web-like protein matrix after mashing (Figure 3.6A). Sections of this matrix were still visible after sonication (Figure 3.6B). This was different from most samples, in that, after sonication only very small pieces of the web-like structures were still visible in the other samples. Figure 3.6C shows the web-like protein matrix after extraction of the residue with reducing agent, in which the matrix was further
degraded. We also observed that the intensity of green areas that represented the quantity of protein matrix became weaker during sequential extraction using sonication and β-ME, indicating more and more proteins were solubilized. CFLSM images confirm that the protein in mashed sorghum was not completely extracted.

**Usefulness of SE-HPLC Profiles for Predicting Ethanol Fermentation**

As reported by Zhao et al (2008), the nine sorghum hybrids used in this study had ethanol yields of 12.36-14.41% (v/v) (13.33% on average) and conversion efficiencies of 83.9-91.1% (87.1% on average). The coefficients of determination ($R^2$) for SE-HPLC area of proteins extracted from those hybrids using sonication and ethanol fermentation are listed in Table 3.1. To make data from different samples with different protein contents comparable, area per milligram of protein was calculated by dividing the SE-HPLC total area or the area of fraction I by the mass of total protein (a product of protein content and mass of the sample used for extraction) in a sample. SE-HPLC total area of proteins from nonmashed sorghum did not correlate to ethanol yield ($p = 0.113$) or conversion efficiency ($p = 0.062$). However, SE-HPLC total area of proteins from mashed sorghum strongly correlated with fermentation parameters ($R^2 = 0.83$, $p = 0.0007$ for ethanol yield and $R^2 = 0.75$, $p = 0.0025$ for conversion efficiency). Ethanol yield increased and conversion efficiency improved notably with the increase in the amount of proteins extracted using sonication (i.e., these factors were highly correlated). The 30-sec sonication disrupted the highly cross-linked proteins in mashed sorghum and greatly enhanced their solubility. Therefore, sonication in combination with SE-HPLC made the protein extraction and quantization process more efficient in terms of precision and time. SE-HPLC total area of proteins extracted from mashed sorghum using sonication could be used as an indicator for predicting fermentation quality of sorghum.

Absolute amount of polymeric proteins (fraction I) extracted using sonication was highly related to ethanol fermentation ($R^2 = 0.67$, $p = 0.007$ for yield and $R^2 = 0.65$, $p = 0.009$ for efficiency). After reduced, the relationship between area of fraction I and fermentation parameters became stronger ($R^2 = 0.83$, $p < 0.0001$ for yield and $R^2 = 0.83$, $p = 0.0007$ for efficiency). SE-HPLC area of extractable non-disulphide cross-linked polymers from mashed sorghum using sonication could also be used as another indicator for fermentation performance.
In conclusion, the amount of proteins extracted using sonication and quantified by SE-HPLC indirectly reflected protein structures that can determine the access of enzymes to sorghum starch and thus could be related to fermentation quality of sorghum.

ACKNOWLEDGMENT

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LITERATURE CITED


Figure 3.1 Comparison of size-exclusion (SE) HPLC total area of proteins extracted from original (nonmashed) and mashed sorghum using sonication. Bars represent standard deviations with four replications. Pooled standard errors were 3,217 for nonmashed samples and 1,694 for mashed samples.
Figure 3.2 Comparison of size-exclusion HPLC separations of proteins extracted from original (nonmashed) and mashed sorghum by sonication in a buffer (12.5 mM sodium borate, pH 10.0, containing 2% SDS) for 30 sec or only with the buffer for 24 hr.
Figure 3.3 Comparison of size-exclusion HPLC separations of proteins extracted from original (nonmashed) and mashed sorghum with different conversion efficiency by sonication in a buffer (12.5 mM sodium borate, pH 10.0, containing 2% SDS) for 30 sec or only with the buffer for 24 hr.
Figure 3.4 Size-exclusion HPLC separations of proteins (extracted from mashed sorghum by sonication) before and after reduction by 2% (v/v) β-Mercaptoethanol (2-ME).
Figure 3.5 Reversed-phase HPLC separations of proteins extracted using 12.5 mM sodium borate, pH 10.0, containing 2% SDS and 2% β-Mercaptoethanol with two extractions of 30 min each from original (nonmashed) and mashed sorghum with different conversion efficiency.
Figure 3.6 Confocal laser-scanning microscopy images (single optical planes) of a sorghum cultivar with low-conversion-efficiency protein matrix (green areas) stained with fluorescein isothiocyanate. A, pellet after mashing; B, pellet after sonication extraction; C, pellet after sequential extraction using sonication and β-Mercaptoethanol.
Table 3.1 Coefficient of Determination ($R^2$) for Size-Exclusion (SE) HPLC Area of Proteins Extracted from nine Sorghum hybrids Using Sonication and Ethanol Fermentation$^a$

<table>
<thead>
<tr>
<th>ethanol fermentation</th>
<th>nonmashed sorghum</th>
<th>mashed sorghum</th>
<th>area of fraction I / mg protein, reduced$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total area / mg protein</td>
<td>total area / mg protein</td>
<td>area of fraction I / mg protein</td>
</tr>
<tr>
<td>ethanol yield$^c$</td>
<td>0.32 $^{ns}$</td>
<td>0.83$^{***}$</td>
<td>0.67$^{**}$</td>
</tr>
<tr>
<td>conversion efficiency$^d$</td>
<td>0.41 $^{ns}$</td>
<td>0.75$^{**}$</td>
<td>0.65$^{**}$</td>
</tr>
</tbody>
</table>

$^a$ SE-HPLC area calculated by dividing integrated area by mass of total protein in a sample. NS, not significant at 5% level; *, **, and ***, significant at 5, 1, and 0.1% level, respectively (from duplicate measurements).

$^b$ Proteins were reduced by 2% β-Mercaptoethanol and fractionated by SE-HPLC after sonication extraction.

$^c$ Amount of ethanol produced (v/v).

$^d$ Calculated from theoretical yield of 56.79 g of ethanol produced from 100 g of dry starch.
CHAPTER 4 - ASSESSING FERMENTATION QUALITY OF GRAIN SORGHUM FOR FUEL ETHANOL PRODUCTION USING RAPID VISCO-ANALYZER

ABSTRACT

The Rapid Visco-Analyzer (RVA) was used to characterize the pasting properties of 68 sorghum grains with a standard 23-min temperature profile. The results showed a strong linear relationship between ethanol yield and final viscosity as well as setback. Ethanol yield increased as final viscosity decreased. A modified RVA procedure (10 min) with an application of α-amylase was developed to simulate the liquefaction step in dry-grind ethanol production. There was a remarkable difference in mashing property among the sorghum samples with the normal dosage of α-amylase. The sorghum samples, which were difficult to liquefy in the mashing step, had much higher peak viscosities than the samples that were easily liquefied. The results also showed that the relationship between conversion efficiency and mashing property was significant. Tannins cause high mash viscosities. There was a strong linear relationship between tannin content and final viscosity as well as peak viscosity. The modified RVA procedure is applicable not only for characterization of mashing properties but also for optimization of α-amylase doses for starch liquefaction.

Keywords: Sorghum; RVA; pasting; amylase; mashing; ethanol; viscosity; tannin

INTRODUCTION

Ethanol production in the U.S. is undergoing an unprecedented expansion. In 2006, a record 4.9 billion gallons of ethanol was produced from 110 biorefineries located in 19 states across the country. This exceeded the previous year’s record by more than 25% (Renewable Fuels Association 2007). Currently, the feedstock for commercial ethanol production is ≈ 97.5%...
corn and 2% sorghum. Sorghum is a drought-resistant, low-input cereal grain, and interest is growing for using it for bioindustrial applications in the United States (Farrell et al 2006). Researchers and ethanol producers have shown that grain sorghum is a reasonable feedstock (e.g., technically acceptable, fits the infrastructure, and can be economically viable) for ethanol and could make a larger contribution to the nation’s fuel ethanol requirements.

In a conventional, dry-grind ethanol process, sorghum is ground and mixed with water to form mash, which is cooked, liquefied, saccharified and fermented to produce ethanol. Mashes used in industry for production of fuel ethanol usually have a dissolved content in the range of 20-24 g/100 mL of mash, and normally a grain-to-water ratio of 1:3 is used (Thomas et al 1995). More recently, fuel alcohol plants have run at alcohol levels as high as 19-20% by volume with the average being nearer to 16-17% (Kelsall and Lyons 2003). Due to the high solids in mash, the viscosity is extremely high during starch gelatinization. In dry-grind processing, thermostable α-amylase enzymes are added as thinning agents to reduce viscosity and partially hydrolyze starch during cooking. Lower mash viscosities improve the heat transfer efficiency in the heat exchangers and allow the plant to process higher levels of dry solids, which significantly reduces energy in heating mash and cooling the cooked liquefact before fermentation. In addition to high energy consumption, high viscosity also may result in incomplete starch gelatinization and low ethanol yield. Therefore, the viscosity of cooked mash could be used as a quality factor for optimizing solids content in the mash, stirring system, and amylase levels.

The Rapid Visco-Analyzer (RVA) has been used to study starch pasting properties through the classic heat-hold-cool profile (Wrigley et al 1996). Compared with wheat and corn, fewer studies have reported on using RVA to study grain sorghum and its associated products. RVA has been mostly used to investigate the pasting properties of isolated sorghum starches or starches in raw, dehulled, decorticated, flaked, and malted sorghum grains, with solid levels of 8.6-14.0% (w/w) in the slurries (Cruzy Celis et al 1996; Moheno-Pérez et al 1997; Taylor et al 1997; McDonough et al 1998; Beta et al 2000; Beta et al 2001; Hugo et al 2000; Suhendro et al 2000; Xie and Seib 2000; Beta and Corke 2001a,b; Agu et al 2006). Previous studies reported RVA procedures with temperature profiles of 13, 18, and 22 min. Beta et al (1995) assayed α-amylase in sorghum malts by measuring the reduction in viscosity using a 3-min rapid test. To simulate an industrial mashing process, Goode et al (2005) successfully used RVA as a rheological tool to characterize the effect of the malt/barley adjunct ratio on viscosity and found
clear correlations between the level of barley adjunct and the RVA parameters. Agu et al (2006) showed that RVA can be used to assess wheat for production of grain whisky and found that RVA peak and final viscosities were highly correlated with alcohol yield.

We hypothesized that sorghum cultivars with high starch contents are generally associated with higher RVA peak and final viscosities that result from larger amounts of gelatinized substrates and produce higher ethanol yields than cultivars with low starch contents. Tannins are well known for effects on inhibition of the $\alpha$-amylase from porcine pancreas (Davis and Hoseney 1979), Bacillus subtilis (Reichert 1980) and Bacillus licheniformis (Wu et al 2007). Measurement of $\alpha$-amylase activity with RVA described by Approved Method 22-08 (AACC International 2000) is based on the ability of $\alpha$-amylase to liquefy a starch gel. Thus, we anticipated that tannins in sorghum should be related to RVA parameters to some extent. To date, published literature contains no reports of using RVA to evaluate tannins in grain sorghum.

Many laboratory dry-grind procedures, all of which belong to the batch cooking system (Kelsall and Lyons 2003), have been developed. For most procedures, fermentation slurry with a first dose of $\alpha$-amylase was cooked at 90-95°C for 45 min or 60 min (Thomas and Ingledew 1990; Ingledew et al 1995, 1999; Thomas et al 1995; Wang et al 1997, 1999; Zhan et al 2003; Wu et al 2006a,b, 2007). After slurry temperature was reduced to 80°C, a second dose of $\alpha$-amylase was added and liquefaction proceeded for an additional 30 min. In some procedures, all of the required $\alpha$-amylase was added to the slurry in one step, and the slurry was cooked at 80°C for 60 min (Lee et al 2000), or at 85°C (Singh and Graeber 2005) or 90°C (Singh et al 2006) for 90 min before the subsequent or simultaneous saccharification step. We assume that a modified RVA procedure, with optimized temperature, time, solids level, and enzyme dosage, could be used to simulate the cooking and liquefaction steps in the dry-grind ethanol process and to quantitatively characterize the mashing properties of sorghum grains.

Therefore, the objectives of this study were to characterize the pasting properties of sorghum grains, simulate the cooking step in a laboratory dry-grind process and identify mashing properties, relate the RVA parameters to ethanol fermentation and optimize $\alpha$-amylase dosage used for fuel ethanol production.

**MATERIALS AND METHODS**
Sorghum Cultivars

A population of 68 sorghum genotypes and elite hybrids, as described in Wu et al (2007) was obtained from the 2004 winter breeding nursery of NC+ Hybrids (Monsanto subsidiary) in Puerto Rico. The samples were hand cleaned to remove glumes, debris, and other impurities, packaged in plastic bags, and stored at 4°C until testing.

Sample Preparation

Tannins in the sorghum samples with pigmented testas were deactivated using the formaldehyde method of Daiber and Taylor (1982) as follows: grain (100 g) of sorghum cultivars was steeped for 6 hr at room temperature in 100 mL of 0.04% (w/v) formaldehyde or distilled water. Grain was then blotted dry and dried at 49°C for 16 hr. The water-steeped samples were used as controls.

Whole kernels (500 g) were decorticated using a tangential abrasive dehulling device (model TADD, Venebles Machine Works Ltd, Sasktoon, Canada) equipped with an 80-grit abrasive pad. The abrasive pad was shimmed to a minimum distance from the upper plate. The decortication level was controlled to ≈20% (by weight) by adjusting the abrasive time.

The original, steeped, and decorticated samples were ground using a mill (Udy Corp., Fort Collins, CO) through a 1.0-mm screen and used for chemical analysis and RVA testing. Samples for ethanol fermentation were ground into fine meals in a grain mill (Magic Mill Products & Appliances, Monsey, NY) set at level III.

RVA Viscosity Measurements

Viscosities of ground sorghum samples during pasting or liquefaction were determined using RVA (model RVA-3D and RVA-4; Newport Scientific, Warriewood, Australia).

For model RVA-3D, a 23-min gelatinization, pasting, and setback profile, as described by Approved Method 76-21 (AACC International 2000), was used. The actual profile is outlined in Table 4.1. Ground samples (4.00 g, 14% wb) were dispersed in 25.00 g of distilled water in aluminum canisters. The RVA parameters measured were pasting temperature, peak time, peak viscosity (maximum hot paste viscosity), holding strength (trough at the minimum hot paste viscosity), and final viscosity (viscosity at the end of the test after cooling to 50°C and holding at this temperature). Breakdown was calculated by the difference between peak viscosity and
holding strength, and setback was defined as the difference between final viscosity and holding strength.

A 10-min liquefaction test was carried out in model RVA-4 as described by Wu et al (2007). The temperature profile was set to maintain a constant block temperature of 95°C for 10 min. An enzyme solution was prepared by diluting 2.30 mL of heat-stable α-amylase (Liquozyme SC DC from Novozymes) to 1 L of distilled water. For most experiments, 1 mL of the enzyme solution containing 2.30 μL of Liquozyme SC DC was added in a canister to liquefy the solids (8.00 g, 14% wb). The enzyme dosage was calculated based on 10 μL of heat-stable α-amylase per 30 g of dry solids in a normal fermentation test. In other experiments, the α-amylase levels in the slurries were multiples (may be greater or less than 1) of the normal dosage. The total weight of water and the required enzyme solution was kept constant at 21.00 g (14% wb). Peak viscosity, peak time, and final viscosity were measured.

Before initiating a sample measurement, a plastic paddle was attached to the stirring head of the RVA and zeroed at 160 rpm against air (Goode et al 2005). After a sample was poured into the water, a plastic paddle was inserted into the sample canister, rotated, and jogged up and down by hand for 15-30 sec to remove lumps. For all RVA measurements, the samples were premixed for 10 sec at 960 rpm, whereafter a speed of 160 rpm was applied. Rheological measurement data were recorded at 4-sec intervals and stored by RVA dedicated software.

**Analytical Methods**

Moisture content was measured using Approved Method 44-15A (AACC International 2000). Total starch content was determined using Megazyme total starch kits according to Approved Method 76-13. Method B was used, which involves pretreatment with dimethyl sulfoxide at 100°C. Amylose content of starch was analyzed by the method of Gibson et al (1997) using an amylose-amylopectin assay kit from Megazyme. Tannin content was evaluated using the modified vanillin/HCl assay of Price et al (1978) with catechin as the standard. Ethanol fermentation was the same procedure as described by Wu et al (2006b).

**Experiment Design**

A split-plot design was used to investigate the effects of tannins on mashing properties. Three sorghum samples (4193, 4194, and 4202), were selected as whole-plot factors. Four sub-
plot factors were 1) decortication, 2) steeping seeds with dilute formaldehyde solution, 3) original untreated seeds, and 4) steeping seeds with distilled water.

**Statistical Analysis**

All experiments were performed at least in duplicate. Tabular results quoted are the mean values of the repeated experiments. Viscosity curves shown represent measurements of one sample. Analysis of variance, least significant difference, split-plot design, and linear regression were performed using SAS software (v.9.1, SAS institute, Cary, NC).

**RESULTS AND DISCUSSION**

**Pasting Properties of Ground Sorghum Grains**

The 23-min standard temperature profile was applied to measure the pasting properties of 68 ground sorghum grains at a solids level of 11.86% (w/w). The numerical data generated by RVA are summarized in Table 4.2. Typical pasting curves selected to represent the 68 sorghum cultivars based on their peak viscosities are displayed in Figure 4.1. Sample 4222 had the lowest peak viscosity (911 cP), while sample 4224 had the highest peak viscosity (3,213 cP). Moreover, these two samples had the lowest two final viscosities (1,193 and 3,159 cP for samples 4222 and 4224, respectively) and the shortest two peak times (7.9 and 7.3 min for samples 4222 and 4224, respectively). Waxy starches or grains were characterized as taking less time to reach maximum viscosities and having lower end viscosities than the nonwaxy counterparts (Cruzy Celis et al 1996; Hayakawa et al 1997; Sasaki et al 2000; Yanagisawa et al 2006). Therefore, Samples 4222 and 4224 were suspected to be waxy cultivars. Amylose analysis verified that amylose contents for samples 4222 and 4224 were 3.0% and 3.2%, respectively. Except for these two waxy cultivars, all of the other 66 sorghum grains gave similar RVA pasting patterns and differed mainly in the magnitude of the viscosities.

The coefficients of determination ($R^2$) for RVA parameters and total starch, ethanol yield, and conversion efficiency are summarized in Table 4.3. As expected, starch contents in ground sorghum grains had a significant effect on peak viscosity, holding strength, breakdown, final viscosity, and setback. Total starch was highly correlated with final viscosity as well as setback ($R^2 = 0.60$, $p < 0.0001$ for final viscosity and $R^2 = 0.55$, $p < 0.0001$ for setback). The relationships between ethanol yield and RVA parameters such as peak viscosity, holding
strength, breakdown, final viscosity, and setback give strong support to the hypothesis that pasting properties of sorghum can be related to ethanol fermentation. There was a strong linear relationship between ethanol yield and final viscosity as well as setback ($R^2 = 0.61$, $p < 0.0001$ for final viscosity and $R^2 = 0.57$, $p < 0.0001$ for setback). However, results of multiple regression showed that the role of starch was dominant ($p < 0.0001$) when combined with final viscosity or setback to predict ethanol yield. Although the effects of pasting properties (peak viscosity, breakdown, final viscosity, and setback) on conversion efficiency were statistically significant ($p < 0.001$), they could only explain $<18\%$ of the variation in efficiency.

Laboratory fermentation tests of 68 sorghum cultivars showed that some samples were easily agglomerated, especially at the beginning of the 45-min liquefaction step when flasks with slurries were directly inserted into a 95°C water bath, becoming difficult to liquefy completely. Representative RVA curves of samples with distinctive liquefaction speeds are shown in Figure 4.2. As reported by Wu et al (2006b, 2007), the two waxy samples (4222 and 4224), were easily handled and liquefied very quickly. Except for 4222 and 4224, it is clear that there were no differences in peak viscosity, holding strength, and breakdown between the two group samples with different liquefaction characteristics. For example, sample 4211 was easily liquefied but had a higher peak viscosity whereas sample 4182 was easily agglomerated but had a lower peak viscosity. Therefore, viscosities measured using the 23-min temperature profile could not be used to explain the difference in mashing characteristics among the sorghum samples.

**Mashing Properties of Ground Sorghum Grains**

In all, 25 sorghum cultivars with broad ranges of starch content, peak viscosity, liquefaction speed, and tannin content were selected for a mashing property study using RVA. The 10-min liquefaction test was programmed to simulate the liquefaction process in a laboratory dry-grind procedure. Solids level in the pasting slurry was 23.72% (w/w), similar to the fermentation test. The dosages of $\alpha$-amylase in the slurries were controlled to be the same if all sound grains had an identical level of endogenous $\alpha$-amylase. A canister with slurry was placed into the heating sink, just like a flask was inserted into a hot water bath in a fermentation test. Starch in the slurry gelatinized almost immediately when the canisters were put into a block that had been pre-heated to 95°C (Figures 4.3-4.5), and viscosity of the slurries increased dramatically. Meanwhile, the heat-stable $\alpha$-amylase tended to reduce viscosity by liquefying the
gelatinized starch. There was a balance between gelatinization and liquefaction that led to peak viscosity. When gelatinization dominated, viscosity increased until reaching the peak value. Viscosity decreased gradually after peaks, with the slurries stirred continuously and the block temperature maintained at a constant of 95°C. Earlier tests showed that, for some samples without tannins, viscosities at a stirring time of 10 min could be reduced to the same levels as achieved at 45 min (data not shown), probably because the hydrolytic action by α-amylase is terminated when the average degree of polymerization is ≈10-12 (Aiyer 2005). To save time, the liquefaction procedure ended at 10 min.

The remarkable difference in mashing properties of sorghum grains with a normal dosage of exogenous enzyme is shown in Figures 4.3-4.5. The difference in mashing properties among sorghum grains suggested that the α-amylase in some samples had been inhibited by some substance, perhaps tannins in those sorghums that contained a pigmented testa (Davis and Hoseney 1979; Reichert 1980). This inhibition could retard the excess of α-amylase to the gelatinized starch, increase peak viscosity, elongate peak time, reduce the rate of viscosity breakdown after peak, and increase final viscosity. It is noteworthy that peak time, peak viscosity, and final viscosity were highly correlated with each other (p < 0.0001), with R² of 0.70 for peak viscosity versus final viscosity, 0.67 for peak viscosity versus peak time, and 0.76 for peak time versus final viscosity.

The 25 samples were divided into 3 groups (Figures 4.3-4.5) according to their peak viscosities. The samples with a slow liquefaction rate in Figure 4.2 were classified into group one (Figure 4.3) and group two (Figure 4.4). All samples with tannins except for 4188 and 4199 belonged to these two groups, suggesting that tannins could be an important factor affecting mashing properties. The samples with a quick liquefaction rate in Figure 4.2 were placed in the third group (Figure 4.5). For all 25 samples, regression analysis showed that starch content did not influence peak viscosity (p = 0.546). Ethanol yield was not affected by peak viscosity (p = 0.099) but was affected by final viscosity (p = 0.038). The relationships between conversion efficiency and mashing properties were negatively significant (R² = 0.33, p = 0.003 for peak viscosity, and R² = 0.30, p = 0.005 for final viscosity). The two waxy samples (4222 and 4224) are among the top 3 grains with the lowest peak viscosities (Figure 4.5), which coincides with our observations in the fermentation tests.
The temperature profile in Figures 4.3-4.5 reflects temperature changes of the block instead of the actual temperature inside the slurries due to heating hysteresis. The block temperature dropped rapidly to ≈85°C after a canister was inserted into the heat sink, but it returned to the 95°C set point in ≈30 sec. Hazelton and Walker (1996) reported that the liquid temperatures did not quite stabilize and were still increasing slightly even at the end of the 3-min test cycle. According to the peak times in Figures 4.3-4.5 (0.13-1.00 min), the estimated peak temperatures could range from 77-92°C based on the results from a 3-min rapid pasting test by Hazelton and Walker (1996).

**Effect of Thermostable \( \alpha \)-Amylase on Mashing Properties**

Based on results shown in Figures 4.3-4.5, two extreme sorghum samples (4222 and 4194) were selected to investigate the effect of \( \alpha \)-amylase on mashing properties. Sample 4222 had the lowest peak viscosity (938 cP), while 4194 had the highest (13,107 cP). The powerful liquefying action of \( \alpha \)-amylase, which lowered viscosity sharply even at very low activity (0.1× normal dosage), is shown in Figure 4.6. All RVA parameters (peak viscosity, peak time, final viscosity, rate of viscosity breakdown after peak, and area under curve) decreased remarkably with increasing levels of heat-stable \( \alpha \)-amylase in the slurries.

\( \alpha \)-amylase was intensively inhibited in the mashes of sample 4194, which had been reported in a recent study (Wu et al 2007). With increasing enzyme activity, peak time and final viscosity decreased slightly. However, peak viscosity did not change significantly (\( p = 0.138 \)), even when the enzyme level was 20× the normal dosage. Sample 4194 was the most difficult to liquefy during mashing in the fermentation tests. Without additional and careful shaking, it was very difficult to disperse and completely liquefy gelled particles.

**Effect of Tannins on Mashing Properties**

For the nine samples with tannins (Figure 4.7), there was a strong linear relationship between tannin content and final viscosity (\( R^2 = 0.91, p < 0.0001 \)) and peak viscosity (\( R^2 = 0.89, p = 0.0001 \)). In addition, peak time was also highly correlated to tannin content (\( R^2 = 0.89, p = 0.0001 \)). These results indicate that RVA could be used to quickly predict tannin contents in sorghum grains.

Because tannins are located in the outer layers, pericarp, and testa of sorghum grain (Reichert et al 1980; Hahn and Rooney 1986), mechanical abrasion of the seed coat layers was
reported to reduce tannin content (Chibber et al 1978). Steeping seeds in dilute formaldehyde solution has been used to decrease tannin content (Daiber and Taylor 1982). Thus, decortication and formaldehyde were applied to remove or inactivate tannins in sorghum grains. Babikir and El Tinay (1993) reported that tannin content can be reduced significantly by steeping whole seeds in water at 30°C. For comparison, the water-steeping method was also used in the present study.

Results of the split-plot design are shown in Table 4.4. Formaldehyde did not react with all tannins in grains, and 23.2-26.3% of total tannins remained after inactivation. Obviously not all outer layers of the seeds were removed, but decortication reduced 88.9-96.0% of total tannins when bran removal was ≈20%. For all 3 sorghum grains, there was no remarkable difference in tannin content between original untreated seed and seed steeped in distilled water. Steeping itself had no effect on tannins.

The inactivation and removal of tannins resulted in significant reduction in peak and final viscosities of sorghum grains (Table 4.4). After such treatments, the sorghum grains with tannins had peak viscosities similar to nontannin grains shown in Figure 4.5. Decorticated samples had slightly larger peak and final viscosities than formaldehyde-steeped samples, possibly due to the higher starch content in decorticated samples (Corredor et al 2006).

Our results showed that samples with extreme high viscosity usually have tannins. However, grains with tannins do not necessarily have problems in liquefaction. For example, tannin content in sorghum 4194 decreased from 39.6 to 10.8 catechin equivalents (CE) (mg/g of sample) after treatment by formaldehyde but it was still higher than that of some other original grains with tannins, such as samples 4188, 4190, 4196 and 4199 (Figure 4.7). However, samples 4188 and 4199 were detected as having tannins (4.00 and 6.59 CE, mg/g of sample, respectively) but had low peak viscosities (Figure 4.5). Davis and Hoseney (1979) reported that condensed tannins in sorghum contain at least two α-amylase inhibition fractions. Presumably not all tannin components were responsible for inhibition of α-amylase. It is also possible that the measured tannin values may come from non-tannin phenolics that reacted with the reagents but were not really tannins (Dykes and Rooney 2006).
Optimization of $\alpha$-Amylase Doses for Mashing

In the dry-grind process, the final mash viscosity after liquefaction reflects the degree of starch hydrolysis by $\alpha$-amylase and thus determines the rate and efficiency of sugar production by amyloglucosidase in the subsequent saccharification, or simultaneous fermentation step. It is very important for fuel producers to efficiently reduce mash viscosity to a proper extent using a sufficient quantity of enzymes. It is also valuable for a producer to use the best kind of liquefying enzymes and optimize enzyme dosages which could result in cost savings.

The difference in final viscosities among sorghum grains when the normal dosage of $\alpha$-amylase was used are shown in Figures 4.3-4.5. The four samples in the first group (Figure 4.3) had the highest final viscosities (414-895 cP). Three samples had final viscosities of 120-150 cP, two of which were sample 4199 and 4210, both having tannins. All others had final viscosities <120 cP (50-119 cP, average 94 cP), which was taken as a reference value for optimization of $\alpha$-amylase levels in this study because the samples with final viscosities less than this value had no problems during mashing in the fermentation tests. Succeeding the encouraging results in Figure 4.6, all 25 grains were subjected to different levels of $\alpha$-amylase in the slurries for the purpose of optimization.

Relationships between final viscosities and levels of $\alpha$-amylase for representative grains are displayed in Figure 4.8. Enzyme levels were expressed as multiples of the normal dosage. When the best-fit curves were applied to the data, clear power correlations were found between final viscosity and $\alpha$-amylase level ($R^2 = 0.991, 0.995, 0.995, 0.981$, for samples 4194, 4202, 4212, and 4222, respectively, with $p < 0.0001$). Different grains fitted different power curves, and most grains behaved like samples 4212 and 4222. The optimized enzyme doses to obtain <120 cP of final viscosities varied among grains. The four samples in Figure 4.3 required more than twice the normal dosage of $\alpha$-amylase, whereas nine samples in Figure 4.5 needed only 50-80% of the normal level. Final viscosities could be reduced to a value of $\approx 40-45$ cP (Figure 4.8), which was the viscosity of a slurry at 60°C before starch gelatinization (data not shown), even with higher enzyme levels.

CONCLUSIONS

The feasibility of using RVA as a tool for assessing the quality of grain sorghum to produce fuel ethanol was investigated in this study. For the 23-min gelatinization, pasting, and
setback profile, there was a strong linear relationship between ethanol yield and final viscosity as well as setback. From this point, RVA could be used as a tool to predict ethanol yield. Sorghum cultivars with higher peak and final viscosities resulting from larger amounts of gelatinized substrates will produce higher ethanol yields than those with low viscosities. The differences in mashing properties among sorghum grains were enlarged and quantified using the 10-min liquefaction test. There was a remarkable difference in mashing properties among representative grains with the normal dosage of α-amylase. It will be very helpful for producers to quickly screen out the grains with abnormal high peak viscosities using RVA. Tannin content was highly correlated to mashing properties. The 10-min RVA procedure could be used as a quick method to predict tannin levels in sorghum grains. For all grains, final viscosities decreased remarkably with increasing levels of heat-stable α-amylase in the slurries. Clear power correlations were found between final viscosities and α-amylase levels. Different grains fitted different power curves, and the optimized enzyme doses to obtain <120 cP of final viscosities varied greatly among grains. These results showed that RVA could be used as a tool to optimize α-amylase doses used for ethanol fuel production. Moreover, RVA could be used for assessment of different commercial enzyme preparations.

ACKNOWLEDGMENTS

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LITERATURE CITED


Figure 4.1 Pasting curves of nine sorghum samples selected from 68 cultivars and measured using the 23-min temperature profile of Rapid Visco-Analyzer model 3D.
Figure 4.2 Pasting curves of 10 sorghum samples selected from 68 cultivars and measured using the 23-min temperature profile of Rapid Visco-Analyzer model 3D. Samples with a slow liquefaction speed are 4182, 4193, 4194, 4202, and 4210 (solid lines); samples with a quick liquefaction speed are 4173, 4211, 4221, 4222, and 4224 (dotted lines).
Figure 4.3 Viscosity curves of four sorghum samples with peak viscosity >7000 cP, measured using the 10-min temperature profile of Rapid Visco-Analyzer model 4 with the normal dosage of α-amylase.
Figure 4.4 Viscosity curves of five sorghum samples with peak viscosity of 4,000-7,000 cP, measured using the 10-min temperature profile of Rapid Visco-Analyzer model 4 with the normal dosage of α-amylase.
Figure 4.5 Viscosity curves of 16 sorghum samples with peak viscosity <4,000 cP, measured using the 10-min temperature profile of Rapid Visco-Analyzer model 4 with the normal dosage of α-amylase. Sample codes are listed from top to bottom according to peak viscosity, starting with the highest value.
Figure 4.6 Viscosity curves of sample 4222 measured using the 10-min temperature profile of Rapid Visco-Analyzer model 4 with increased levels of α-amylase in the slurries. The normal dosage of α-amylase contained 2.30 μL of Liquozyme SC DC for liquefying the solids (8.00 g, 14% wb) in a canister.
Figure 4.7 Relationships between tannin content and peak and final viscosity measured using the 10-min temperature profile of Rapid Visco-Analyzer model 4 with the normal dosage of α-amylase.
Figure 4.8 Effects of α-amylase levels on final viscosities of different sorghum grains measured using the 10-min temperature profile of Rapid Visco-Analyzer model 4. The normal dosage of α-amylase contained 2.30 μL of Liquozyme SC DC for liquefying the solids (8.00 g, 14% wb) in a canister.
Table 4.1 A Standard 23-min Gelatinization, Pasting, and Setback Profile

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Table 4.2 Rapid Visco-Analyzer (RVA) Test Results for Sorghum Samples Using the 23-min Temperature Profile of RVA-3D

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<th>breakdown (cP)</th>
<th>final viscosity (cP)</th>
<th>setback (cP)</th>
<th>peak time (min)</th>
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Table 4.3 Coefficient of Determination ($R^2$) for Rapid Visco-Analyzer (RVA) Parameters and Total Starch, Ethanol Yield, and Conversion Efficiency$^a$

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</tr>
<tr>
<td>ethanol yield</td>
<td>0.47***</td>
<td>0.27***</td>
<td>0.36***</td>
<td>0.61***</td>
<td>0.57***</td>
<td>0.0017ns</td>
<td>0.0043ns</td>
</tr>
<tr>
<td>conversion efficiency</td>
<td>0.16***</td>
<td>0.05NS</td>
<td>0.17***</td>
<td>0.18***</td>
<td>0.18***</td>
<td>0.0003ns</td>
<td>0.0005ns</td>
</tr>
</tbody>
</table>

$^a$ RVA parameters were measured using the 23-min temperature profile of RVA-3D; *** significant at 0.1% level; ns, not significant at 5% level.
Table 4.4 Tannin Inactivation by Formaldehyde and Removal by Decortication and Effects of Tannin on Sorghum Mashing Properties Measured Using the 10-min Temperature Profile of Rapid Visco-Analyzer model 4 with Normal Dosage of α-amylase

| sample code | treatment description                  | tannin content (catechin equivalent, mg/g)
|-------------|---------------------------------------|---------------------------------------------|
|             |                                       | peak viscosity (cp)
|             |                                       | final viscosity (cp)                        |
| 4193        | decorticated with 19.9% bran removal   | 0.8 ± 0.1A                                  | 2398 ± 45Aa                               | 183 ± 8Aa                     |
|             | steeped in 0.04% formaldehyde for 6 h  | 4.6 ± 0.2B                                  | 2296 ± 31Aa                               | 112 ± 3Ab                     |
|             | original, untreated                    | 19.8 ± 1.4C                                 | 7781 ± 582Ba                              | 439 ± 21Ba                    |
|             | steeped in distilled water for 6 h     | 20.4 ± 0.7C                                 | 8331 ± 763Ba                              | 426 ± 27Ba                    |
| 4194        | decorticated with 20.4% bran removal   | 4.4 ± 0.1A                                  | 4234 ± 79Aa                               | 134 ± 4Aa                     |
|             | steeped in 0.04% formaldehyde for 6 h  | 10.8 ± 0.9B                                 | 2742 ± 61Bb                               | 110 ± 3Ab                     |
|             | original, untreated                    | 39.6 ± 1.5C                                 | 13107 ± 878Ca                             | 895 ± 88Ba                    |
|             | steeped in distilled water for 6 h     | 39.0 ± 0.9C                                 | 13721 ± 803Ca                             | 681 ± 54Cb                    |
| 4202        | decorticated with 22.9% bran removal   | 2.6 ± 0.4A                                  | 4965 ± 90Aa                               | 110 ± 7Aa                     |
|             | steeped in 0.04% formaldehyde for 6 h  | 6.1 ± 0.2B                                  | 4413 ± 55Ab                               | 90 ± 7Ab                      |
|             | original, untreated                    | 25.0 ± 0.7C                                 | 11401 ± 754Ba                             | 414 ± 30Ba                    |
|             | steeped in distilled water for 6 h     | 25.0 ± 0.3C                                 | 12405 ± 733Ba                             | 322 ± 17Bb                    |

*a Mean ± standard deviation of triplicate measurements for tannin contents; means followed by the same uppercase letter for the same sample in the same column are not significantly different (p < 0.05).

*b Mean ± standard deviation of duplicate measurements for RVA viscosities; means with the same lowercase letter for the first two or the last two treatments applied to the same sample in the same column are not significantly different (p < 0.05).
CHAPTER 5 - SMALL-SCALE MASHING PROCEDURE FOR PREDICTING ETHANOL YIELD OF SORGHUM GRAIN 4

ABSTRACT

A small-scale mashing (SSM) procedure requiring only 300 mg of samples was investigated as a possible method of predicting ethanol yield of sorghum grain. The initial SSM procedure, which was conducted similarly to the mashing step in a traditional fermentation test, hydrolyzed just 38.5-47.2% of total sorghum starch to glucose. The initial procedure was simplified to contain only one liquefaction step, which did not influence subsequent saccharification. Thereafter, parameters such as temperature, pH, enzyme dosage, and saccharification time were optimized. Results showed that 91.2-97.5% of the total starch in 18 sorghum hybrids had been hydrolyzed to glucose using the following conditions: liquefaction at 86°C for 90 min, 20 µL of α-amylase per 30 g of sample; pH adjustment by adding 50 µL of 2 M acetate buffer at pH 4.2 to each microtube; saccharification at 68°C for 90 min, 200 µL of α-amylglucosidase per 30 g of sample. There were strong linear correlations between completely hydrolyzed starch (CHS) from SSM and ethanol yields from both traditional (R² = 0.86) and simultaneous saccharification and fermentation (SSF, R² = 0.93) procedures. CHS was a better indicator for predicting ethanol yield in fermentation than total starch.

Keywords: Sorghum; Starch; Mashing; Glucose; Ethanol; Fermentation; SSF; HPLC

INTRODUCTION

Promoting petroleum alternatives, including fuel ethanol, is an ongoing goal of U.S. energy policy. Federal incentives for ethanol use, such as tax incentives, the reformulated gasoline oxygenate standard, and the renewable fuels standard (RFS), promoted significant growth in the ethanol market. The new RFS schedule in law H.R. 6, the "Energy Independence and Security Act of 2007", calls for a minimum of 9 billion gallons of renewable and alternative

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fuels to be used nationwide in 2008, 20.5 billion gallons by 2015, and 36 billion gallons by 2022. U.S. ethanol production capacity reached 7.8 billion gallons by the end of 2007, a 44% increase from 2006. National production capacity will increase to 13.4 billion gallons at 139 facilities in 2008 if all existing projects are completed (Renewable Fuels Association, 2008).

Grain sorghum is a reasonable feedstock for ethanol and could make a larger contribution to the nation’s fuel ethanol requirements. Interest in using grain sorghum for bio-industrial applications is growing in the United States (Farrell et al., 2006). There is a large variation in fermentation quality among the hundreds of sorghum hybrids available commercially and under development; thus, it is important for the ethanol industry and sorghum producers to have proper methods that accurately predict sorghum ethanol yields and conversion efficiencies. Little research has been conducted to develop methods for evaluating sorghum fermentation quality.

Ethanol yield and conversion efficiency are major quality traits of cereal grains used as feedstocks to produce fuel ethanol. Grains with higher ethanol yield per unit will produce larger volumes of final product. Although laboratory fermentation is the most direct and reliable method of evaluating fermentation quality of cereal grains, current dry-grind procedures are time-consuming, tedious (Ingledew et al., 1995, 1999; Lee et al., 2000; Singh and Graeber, 2005; Singh et al., 2006; Thomas and Ingledew, 1990; Thomas et al., 1995; Wang et al., 1997, 1999; Wu et al., 2006a,b, 2007; Zhan et al., 2003), and require relatively large quantities of samples.

The dry-grind procedure is a biological process in which starch in whole grains is hydrolyzed to fermentable sugars such as glucose, maltose, and maltotriose, and sugars are converted into ethanol and carbon dioxide. Thus, the amount of total starch in grains is thought to be related to ethanol yield. Previous research in our group showed that starch content in sorghum is a good indicator of ethanol yield in the dry-grind process (Wang et al., 2008; Wu et al., 2007; Zhan et al., 2003). However, not all starch could be completely converted to fermentable sugars by enzymes. For example, the web-like protein matrix developed during mashing held not only starch granules but also some oligosaccharides or polysaccharides (Wu et al., 2007; Zhao et al., 2008). Incomplete starch gelatinization and inaccessibility of enzymes to the gelatinized starch also limit the ability to predict ethanol yield from total starch.

In AACC Approved Method 76-13 (AACC International, 2000), starch is hydrolyzed by sequential treatment with thermostable α-amylase and amyloglucosidase. This enzymic procedure requires high-purity enzymes, especially amyloglucosidase, free of contaminating

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activities from cellulase and catalase. Cellulase contamination contributes to false high starch values due to cellulose hydrolysis, and catalase reduces stability of chromogen formed in glucose assay methods based on the use of GOPOD reagent (McCleary et al., 1997). Megazyme (Megazyme International Ireland Ltd., Ireland) offers a total starch assay kit based on thermostable $\alpha$-amylase and amylloglucosidase. However, widespread use of this approach in industry is limited by the prohibitive cost for per-sample analysis.

According to AACC Approved Method 76-13 (AACC International, 2000), complete solubilization and dextrinization for samples containing enzyme-resistant starch requires pre-treatment with dimethyl sulphoxide (DMSO) at boiling temperature. Results from sorghum samples using Megazyme kits showed that total starch contents with DMSO pre-treatment were slightly higher than for samples without DMSO (unpublished data). Pre-treatment with DMSO is favorable and necessary to avoid underestimating starch content in sorghum samples. However, it is impossible for ethanol fermentation practices to have a DMSO pre-treatment before liquefaction and saccharification, which could be one reason that total starch content explained only 78% of the variability of ethanol yield among 70 sorghum samples (Wu et al., 2007). Completely converting starch to glucose in a short time requires enzyme levels much higher than those used in ethanol fermentation for the quantified amount of substrates. This could affect total starch content to explain the variability of ethanol yield among sorghum samples.

Research from Pioneer, a seed company, showed that the high total fermentables (HTF) trait of corn is a more accurate indicator of dry-grind ethanol production than total starch (Bryan, 2003). Additionally, Pioneer developed a point-of-sale assay using whole grain near-infrared (NIR) technology that allows ethanol plants to predict the value of corn for ethanol production by identifying HTF grain arriving at the plant.

The aim of this study was to develop a small-scale mashing (SSM) procedure that is similar to the mashing step in a fermentation test but requires only a few hundred milligrams of test samples. Industry-used enzymes including heat-stable $\alpha$-amylase and amylloglucosidase would be employed in SSM as in fermentation procedures. We expected most of the starch in ground sorghum to convert to glucose with optimized liquefaction and saccharification parameters and hypothesized that ethanol yield from laboratory fermentation would be highly correlated to glucose yield from SSM. Samples would not be pre-treated with DMSO, and the effect of starch granules firmly trapped within the web-like protein matrix (Zhao et al., 2008) on
glucose yield could be similar to their effect on ethanol yield. Glucose yield from SSM was anticipated to be a better indicator than total starch content for prediction of ethanol yield in fermentation.

A mini shaking incubator made it possible to develop the SSM procedure and was used in this study. The incubator was a kind of vortex mixer with accurate temperature control, and small samples could be simultaneously heated and mixed. Thus, we anticipated that the combination of an SSM procedure with rapid glucose determination in a mash would make prediction of ethanol yield more efficient. Moreover, the cost for per-sample analysis would be much lower than the total starch assay.

EXPERIMENTAL

Sorghum Cultivars

Eighteen cultivars from a 2004 commercial winter breeding nursery were selected from a population of 70 proprietary sorghum genotypes and elite hybrids with a broad range of ethanol yields. Samples were hand cleaned to remove glumes, debris, and other impurities, packaged in plastic bags, and stored at 4°C until testing.

Sample Grinding

For SSM, samples were ground using a Udy mill (Udy Corp., Fort Collins, CO) through a 0.25-mm screen. For starch analysis, sorghum kernels were ground using the Udy mill with a 1.0-mm screen. Samples for ethanol fermentation were ground into fine meals in a Magic Mill III Plus grain mill (Magic Mill Products & Appliances, Monsey, NY) set at level III.

Enzymes and Microorganisms

Liquozyme SC DC, a heat-stable $\alpha$-amylase from *Bacillus licheniforms* was used for liquefaction. Enzyme activity was 240 KNU/g (KNU, the amount of enzyme which breaks down 5.26 g of starch per hour by Novozyme’s standard method for determination of $\alpha$-amylase). Spirizyme Fuel, an amyloglucosidase from *Aspergillus niger*, was used for saccharification. Enzyme activity was 750 AGU/g (AGU, the amount of enzyme which hydrolyzes 1 μmol of maltose per minute under specified conditions).
Saccharomyces cerevisiae (ATCC 24860) was used for traditional fermentation. Yeast cells were maintained on yeast extract/peptone/dextrose medium. The agar slant consisted of yeast extract (20 g/L), peptone (5 g/L), dextrose (5 g/L), agar (20 g/L), and distilled water. Yeast cells were precultured for 48 h at 30ºC in an aqueous solution containing glucose (20 g/L), peptone (5 g/L), yeast extract (3 g/L), KH$_2$PO$_4$ (1 g/L), and MgSO$_4$$\cdot$7H$_2$O (0.5 g/L) with a shaking speed of 200 rpm. Yeast cell counts were determined by the direct microscopic method at a magnification of 400× using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA) with a Micromaster phase-contrast microscope (Fisher Scientific, Fairlawn, NJ). Cell concentrations also were checked by measuring the optical density of the yeast precultures at a wavelength of 600 nm on a BioRite 3 spectrophotometer (Thermo Electron Corporation, Madison, WI). The A$_{600}$ values of the 48 h precultures were 2.2-2.6 with cell counts of 2-2.8 × 10$^8$ cells/mL.

Active dry $S.\,\,cerevisiae$, Red Star Ethanol Red, was used for simultaneous saccharification and fermentation (SSF). Before inoculation, dry yeast was activated by adding 1.0 g of cells into 19 mL of the same preculture broth described previously and incubated at 38°C for 25-30 min in an incubator operating at 200 rpm. The activated yeast culture had a cell concentration of 1×10$^9$ cells/mL.

**Preparation of Mashes for Ethanol Fermentation**

For mashing, 30 g (db) of ground sorghum was dispersed in a 250-mL Erlenmeyer flask with an aliquot of 100 mL of fermentation solution, which was prepared by mixing 1 L of distilled water (60-65°C) with 1.0 g of KH$_2$PO$_4$, 3.0 g of yeast extract, and 100 μL (for two-step liquefaction) or 200 μL (for one-step liquefaction) of Liquozyme. Flasks then were inserted into a water bath shaker (Amerex Instruments, Inc., Lafayette, CA) oscillating at 100 rpm. The water bath had been preheated to 95°C. In the beginning, flasks were shaken manually to prevent gel formation. Some grains had to be shaken intensively. This shaking process took several minutes depending on the number of flasks inserted. The water bath temperature decreased naturally to 82-87°C at the end of shaking, and slurries in the flasks were well dispersed. Liquefaction proceeded in two different ways as follows:

**One-step Liquefaction.** The temperature was brought to 86°C, and slurries were incubated at this temperature for 90 min in the water bath shaker at 100 rpm.
Two-step Liquefaction. The temperature was raised to 95°C and held for 45 min with continuous shaking. Flasks then were taken out of the water bath and cooled to 80°C, and another 10 μL of Liquozyme was added to each flask. Liquefaction continued for an additional 30 min at 80°C.

At the end of liquefaction, flasks were taken out of the water bath, and the materials sticking on the inner surface of the flasks were scraped back into the slurries with a spatula and rinsed with 3-5 mL of deionized distilled water using a sterilized, fine-tipped polyethylene transfer pipette. Only slurries after two-step liquefaction were saccharified separately before fermentation.

Saccharification. The temperature of the liquefied slurries was lowered to 60°C, and 100 μL of Spirizyme was added to each flask. Flasks were maintained at 60°C for 30 min with the shaker running at 100 rpm.

Fermentation Processes

All of the mashes after one-step liquefaction and the saccharified mashes were cooled to 25-30°C and adjusted to pH 4.2-4.3 with 2M HCl before inoculation.

Traditional Fermentation. Saccharified mashes were inoculated with 5 mL of yeast preculture (S. cerevisiae ATCC 24860).

SSF. For one-step liquefied mashes, SSF started with addition of 1.0 mL of the activated dry yeast culture and 100 μL of Spirizyme to each flask.

Fermentation flasks were sealed with S-shaped airlocks filled with ≈2 mL of mineral oil. Ethanol fermentation was performed in an incubator shaker (Model 12400, New Brunswick Scientific Inc., Edison, NJ) at 30°C for 72 h with continuous shaking at 200 rpm. The fermentation process was monitored by measuring the mass loss of the fermentation mash.

Distillation

At the final fermentation time (72 h), all materials in a flask were transferred to a 500-mL distillation flask with 100 mL of distilled water. Beers were distilled on a distillation heating unit, and the distillates were collected into a 100-mL volumetric flask that was dipped into ice water. Distillation was stopped when the collected distillates approached the 100-mL mark (≈ 99 mL). Collected distillates then were equilibrated to 25°C, adjusted to 100 mL, and sampled for HPLC analyses.
Preparation of Freeze-dried Mashes

Ground sorghum (30 g, db) was liquefied by Liquozyme at 95°C for 45 min and 80°C for 30 min and then saccharified by Spirizyme at 60°C for 30 min. After mashing, all materials in each flask were collected, frozen immediately, and then lyophilized. Enzyme dosages and mashing conditions were the same as preparation of mashes for ethanol fermentation, but yeast foods such as KH₂PO₄ and yeast extract were excluded from the mashes. All mashed grains were ground using the Udy mill with a 0.25-mm screen.

SSM Experiments

A mini shaking incubator (Vortemp 1550, Labnet International, Inc., Edison, NJ) with a shaking rack for microtubes was used for SSM. Speed of the shaking platform was set at 900 rpm for all procedures. Before mashing, ground meal (300 ± 5 mg, as is basis, weighted accurately) was mixed with 1 mL of enzyme solutions in a 2-mL pre-weighted microtube, and the microtube was vortexed to disperse the flour. Centrifuge microtubes were tolerant of high mashing temperatures (e.g., 100°C) and tightly-sealed during mashing. Enzyme solutions were prepared fresh by diluting Liquozyme or Spirizyme to a desired concentration in distilled water. For comparative purposes, the enzyme level in each microtube was calculated as the amount of an enzyme, Liquozyme or Spirizyme, which was used to liquefy or saccharify the substrate in 30 g of ground sorghum. Various dosages of Liquozyme (5, 10, and 20 µL per 30 g of sample) were used for liquefaction. Microtubes with slurries were inserted in the shaking rack, which then was put into the incubator at room temperature. Liquefaction proceeded in two different ways as follows:

One-step Liquefaction. The incubator temperature was raised to 86 or 95°C and held for 90 min.

Two-step Liquefaction. The incubator temperature was raised to 95°C and held for a period of time (45 or 60 min). The shaking rack together with the microtubes then was taken out and cooled for 5 min at room temperature while the incubator cooled naturally with its lid open. One hundred µL of an α-amylase dilution was added to each microtube with the second dosage equivalent to 10 µL of Liquozyme per 30 g of sample. Microtubes were vortexed and re-placed in the incubator. Incubator temperature was brought to 80 or 86°C and held for 30 or 45 min.
After liquefaction, the shaking rack together with the microtubes was taken out, and the incubator cooled naturally with its lid open. In some cases, the pH of the liquefied mashes was adjusted by adding 50 μL of 2 M sodium acetate buffer at pH 3.5, 4.2, 4.5, or 5.5 to a microtube. For saccharification, 100 μL of a properly diluted amyloglucosidase solution was added to each microtube with the dosage equivalent to 15, 50, 100, 150, 200, or 250 μL of Spirizyme per 30 g of sample. Microtubes were vortexed and then put into the incubator.

**Saccharification.** The incubator temperature was raised to 60 or 68°C and held for a period of time (30, 45, 60, 90, or 120 min).

After mashing, microtubes were cooled at room temperature for 20 min. During cooling, microtubes were vortexed and weighed. All mashes were centrifuged at 13,200 × g for 4 min, and the supernatants were filtered through syringe filters with a 0.45-μm membrane.

**Mash Dilution and Inactivation of Amyloglucosidase.** The mash was diluted by mixing 200 μL of the filtrate with 480 μL of distilled water. In some cases, dilutions were sealed in 15-mL test tubes and cooked at 100°C for 10 min to deactivate amyloglucosidase. In most cases, the mash was diluted by mixing 200 μL of the filtrate with 480 μL of 0.01M phosphate buffer at pH 10.0 for inactivation of amyloglucosidase. The phosphate buffer was prepared by dissolving 59.8 mg of NaH₂PO₄ and 190.7 mg of Na₃PO₄•12H₂O with distilled water to 1 L using the phosphate buffer calculator (Clymer, 2005). Some uncooked and cooked dilutions were kept at room temperature for 1-3 days. All dilutions were sampled for HPLC analyses.

**Analytical Methods**

Moisture content was measured using AACC Approved Method 44-15A (AACC International, 2000). Total starch content was determined using a Megazyme total starch kit (Megazyme International Ireland Ltd., Wicklow, Ireland) according to AACC Approved Method 76-13. Method B was used, which involves pretreatment with DMSO at 100°C. For glucose analysis in freeze-dried mashes, 0.5 g of samples was dispersed in 10 mL of H₂O. After vortexing for 5 min, the slurry was diluted to 250 mL, and glucose in the diluted solution was measured following the total starch method and analyzed by HPLC.

Sugars (glucose, maltose, and maltotriose) in diluted mashes from SSM and ethanol in distillate samples from fermentation were determined using a Shimadzu (Shimadzu Scientific Instruments, Inc., Columbia, MD) HPLC system equipped with a Rezex RCM 7.8×300 mm
column (Phenomenex, Torrance, CA) with a security guard column. The mobile phase used was deionized distilled water at a flow rate of 0.6 mL/min. Injection volume was 20 μL. All components were detected with a refractive index detector (Model RID-10A, Shimadzu). Temperatures of the column and detection cell were maintained at 80°C and 40°C, respectively. To determine sugar and ethanol concentrations, HPLC data were processed using EZStart 7.4 software (Shimadzu).

Completely hydrolyzed starch (CHS) (% db) was defined as a ratio of the mass of the starch that had been hydrolyzed to glucose in a mash to the sample mass (dry matter).

**Experimental Designs**

As detailed in Table 5.1, four split-plot designs were used to investigate the effects of SSM conditions on CHS, and two completely random designs were used to optimize enzyme dosages and saccharification time.

**Statistical Analyses**

All experiments were performed at least in duplicate. The tabular and diagrammatic results presented are the mean values of the repeated experiments. Analysis of variance, least significant difference (LSD), split-plot design, and linear regression were performed using SAS software version 9.1 (SAS institute, Cary, NC).

**RESULTS AND DISCUSSION**

**Calculation of the Mass of Glucose and the CHS in a Microtube**

To calculate the percentage of the starch which had been hydrolyzed to glucose in a sample, it was necessary and crucial to determine the mass of glucose in a microtube after SSM. Glucose concentration in a mash (expressed as mass of glucose per milliliter of solution) can be assayed conveniently by HPLC or the enzymic method (McCleary et al., 1997). However, measuring the total volume of the liquid part of the mash is difficult due to the concentrative properties of glucose aqueous solutions, in which the volume of solution is larger than that of water solvent. The most accurate way to obtain the mass of glucose is diluting all of the mash in a microtube to a known volume and then analyzing the glucose concentration in the dilution.
This approach was time-consuming and was used only as a control for the measurement of glucose mass in this study.

Several sources of water including the water in a sample, diluted enzyme solutions, and the buffer for pH adjustment, contributed to the mass of water brought to a microtube after mashing. Mass of water in a mash was not equal to the sum of the sub-masses of all water sources due to evaporation, but it could be calculated using the following equation:

\[ M_w = M_{tm} - M_s - M_t + M_s \times \frac{MC}{100} \]  

where, \( M_w \) = mass of water (mg), \( M_{tm} \) = mass of a microtube together with mash after mashing (mg), \( M_s \) = mass of a sample (mg), \( M_t \) = mass of the empty microtube (mg), and \( MC \) = moisture content of the sample (%).

Looking at the concentrative properties of glucose aqueous solutions at 20°C (Anonymous, 2004), we found that there was a strong, linear relationship \((R^2 = 1.00, p < 0.0001)\) between the two concentrations, mass of glucose per milliliter of glucose solution \((C_1, \text{mg/mL})\) and mass of glucose divided by total mass of glucose solution \((C_2, \%)\). \( C_1 \) was transformed from the molality (moles of glucose per liter of solution) by multiplying by 180. Thus, Eq. (2), the regression equation, was used to calculate mass percentage of glucose in a mash solution as follows:

\[ C_2 = 0.0928 \times C_1 + 0.2352 \]  

For simplicity, we assumed that the liquid part of the mash in a microtube was an aqueous solution of glucose (i.e., most of the starch had been hydrolyzed to glucose). This assumption was proved in SSM with optimum parameters and is discussed later. Based on the definition of mass percentage \((C_2)\), the mass of glucose in a microtube at room temperature (22-24°C) could be calculated using the following equation:

\[ M_g = \left( \frac{C_2}{100 - C_2} \right) \times M_w \]  

where, \( M_g \) = mass of glucose (mg).

CHS (% db) was calculated using the following equation:

\[ CHS = \left( \frac{M_g \times (162/180)}{M_s \times (100 - MC)} \right) \times 10,000 \]
where, \( \frac{162}{180} \) = adjustment from free glucose to anhydro glucose (as occurs in starch) and \( 10,000 \) = factor to express CHS as a percentage of the sample mass.

**Initial SSM Procedure**

At the beginning of this research, we tried an SSM procedure that followed the traditional fermentation process: liquefaction at 95°C for 45 min and 80°C for 30 min, \( \alpha \)-amylase dosages equivalent to \( 2 \times 10 \) µL of Liquozyme per 30 g of sample; saccharification at 60°C for 30 min, amylglucosidase equivalent to 100 µL of Spirizyme per 30 g of sample. A typical chromatogram of a mash analyzed right after diluting by distilled water is shown in Figure 5.1. Results of sugar analyses by HPLC for the nine sorghum samples (Table 5.2) showed that 38.5-47.2%, 21.1-27.4%, and 1.7-6.7% of the total starch had been hydrolyzed to glucose, maltose, and maltotriose, respectively (data not shown). HPLC could not separate oligosaccharides and polysaccharides with glucose units greater than three, but a group of starch hydrolyzates with degree of polymerization (DP) >3 were present in the mash and represented 21.2-35.4% of the total starch (calculated from the difference between total starch and sum of the starch which had been hydrolyzed to glucose, maltose, and maltotriose). Existence of maltose, maltotriose, and other hydrolyzates influences calculation of the mass of glucose in a mash. Using the earlier developed procedure, the mass of glucose was 5-10% lower than that in direct measurement after the mash in a microtube had been diluted to a known volume (data not shown), which was one reason the initial procedure needed to be modified further.

Glucose concentrations for the same mash dilution were slowly but continuously increased when measured repeatedly over time because amylglucosidase in the diluted mash remained active throughout the room temperature setting. During a 3-day observation with the same mash sample, the peak of glucose increased while all other peaks decreased (Figure 5.1). The hydrolysis of starch hydrolyzates with higher DP by amylglucosidase to glucose was a dynamic process. However, this process seemed to cease after 3 days; chromatograms were unchanged and appeared similar to the one with a dotted line in Figure 5.1. To improve experimental repeatability and guarantee the analysis results were not affected by the setting time after mashing, it was necessary to deactivate amylglucosidase in the mash dilutions.

In SSM, slurries in microtubes were heated in air using a mini shaking incubator. In mashing procedures for fermentation, slurries in flasks were heated in a water bath shaker. Due
to heat transfer, temperature of the slurries in the microtubes will lag behind that of the slurries in the flasks if both the incubator and the water bath are preheated to the same temperature (e.g., 95°C). As listed in Table 5.2, it took an extra liquefaction time (15 min at 95°C) and an extra saccharification time (15 min at 60°C) for mashes from SSM to have CHS similar to mashes prepared for laboratory fermentation. It was feasible for the SSM procedure to simulate the mashing process in a laboratory fermentation test. Freeze-dried mashes had slightly higher CHS than mashes from SSM, probably due to the continuous activity of amyloglucosidase in the process of cooling and freezing.

As shown in Table 5.2, only 49.8-58.6% of the total starch had been completely hydrolyzed to glucose for the nine sorghum samples with SSM (calculated by dividing the values in the third column by their counterparts in the second column). Moreover, there was no significant correlation between CHS from SSM and ethanol yield from traditional fermentation ($R^2 = 0.10, p = 0.41$). The difference in CHS among the samples could not explain the variability in ethanol yield, another reason the initially developed SSM procedure needed to be modified.

**Inactivation of Amyloglucosidase**

According to manufacturer's instructions, the optimum temperature range for amyloglucosidase, Spirizyme Fuel, was 65-70°C (Novozymes, 2004a), but surprisingly, this enzyme still worked well at room temperature even after heated at 60°C for 45 min and cooked at 100°C for 10 min (Figure 5.2). Amyloglucosidase was not sensitive to saccharification temperature and was active throughout fermentation. Thus, the heat-stability characteristic of amyloglucosidase is beneficial for ethanol production. The deactivating effect of boiling on amyloglucosidase was significant; CHS values after treatment II-3 were statistically lower ($p < 0.0001$) than those after treatment I-3. However, boiling was neither an efficient nor simple way to inactivate amyloglucosidase.

Amyloglucosidase was effectively deactivated by diluting 200 μL of mash supernatant with 480 μL of 0.01 M phosphate buffer at pH 10.0 (Figure 5.2). CHS values of both samples in treatment III did not change significantly over time (0-3 days) after mash dilution ($p > 0.46$). Again, CHS values in treatment I-0 (control) were slightly higher than those in treatment III ($p < 0.0001$) due to the short enzymic reaction time starting from preparation of mash dilution to sugar analysis by HPLC.
Effect of pH Adjustment on SSM

The optimum pH range for efficient usage of Liquozyme was 5.7-6.0 (Novozymes, 2004b). The pH values of the slurries, mixtures of ground grains and water containing no backset stillage, ranged from 6.0 to 6.3 (data not shown). For liquefaction, we did not adjust pH in our fermentation procedures or in SSM.

In the traditional fermentation procedure, we adjusted the saccharified mashes to pH 4.2-4.3 with 2M HCl before yeast inoculation. There was no pH adjustment for the separate saccharification, in which Spirizyme did not hydrolyze the substrates under its optimum pH of 3.5-4.5 (Novozymes, 2004a). Similar to the previously described results for SSM, about 51.2-62.7% of the total starch had been completely hydrolyzed to glucose in the freeze-dried mashes (Table 5.2). For the SSF procedure, we expected much lower levels of glucose in the liquefied mashes before inoculation. However, final fermentation results were not affected due to the continuous activity of amyloglucosidase during fermentation.

Compared with the fermentation procedure, it was not as convenient to insert a normal pH meter probe into a mash in SSM. Cleaning the electrode of a pH meter with minimal influence on the volume of a mash would be a challenge. In addition, titration with an acidic solution would be time-consuming and tedious, as found in preparation of fermentation broths. Considering these factors, the 2 M sodium acetate buffer at pH 4.2 was attempted, 50 μL of which was added directly to the microtube after liquefaction to adjust the pH of the mash for saccharification. This approach originated from the total starch assay (AACC International, 2000), in which 200 mM acetate buffer at pH 4.5 was used for pH adjustment. For each microtube, final ionic strength in the mash was about 80-90 mM of sodium acetate, which was similar to that in the total starch assay (80-110 mM).

Obviously in Figure 5.3, amyloglucosidase was the most important determinant in converting starch hydrolyzates to glucose after liquefaction. Little glucose existed in the liquefied mashes (only 1.2% of CHS for both samples). The difference in CHS between treatment II and III was significant (p < 0.0001), suggesting that pH was important for saccharification by amyloglucosidase in SSM. Without pH adjustment (treatment II), the two samples had no statistical difference in CHS (p = 0.08), but they were significantly different in treatment III (p = 0.002). Thus, pH adjustment was expected to increase the resolution of SSM in differentiating samples.
Simplification of SSM Procedures

Because they varied in the liquefaction step, three SSM procedures (1, 2, and 3) were tested further (Table 5.3). Both 95°C and 86°C were selected as the liquefaction temperature in consideration of starch gelatinization in sorghum grain and the optimum temperature range of 82-86°C for Liquozyme (Novozymes, 2004b). Similar to the initially developed SSM procedure, the two-step liquefaction structure was kept in Procedure 2, but the temperature in the second step was increased to 86°C. Liquefaction time for each step was 45 min. Procedures 1 and 3 were simplified from Procedure 2; they had the one-step liquefaction structure and a total 90 min of liquefaction time. Liquefaction temperatures were 95°C and 86°C in Procedures 1 and 3, respectively. In all procedures, saccharification temperature was raised to 68°C in accordance with the optimum temperature of 65-70°C for Spirizyme (Novozymes, 2004a), and saccharification time also was extended to 90 min for the purpose of obtaining higher CHS. The α-amylase dosage was equivalent to a total 20 μL of Liquozyme per 30 g of sample, except in procedure 2, where half the dosage was used for each step. The pH of the liquefied mash was adjusted by adding 50 μL of 2 M sodium acetate buffer at pH 4.2 to each microtube, and amyloglucosidase in the mash dilution was deactivated with 0.01 M phosphate buffer at pH 10.0 before sugar analysis.

There was no significant difference in CHS between Procedure 2 and 3 (p = 0.22), indicating that one-step liquefaction would be feasible (Table 5.3). Procedure 1 had systematically lower CHS than the other two procedures (p < 0.02), more than likely due to the harsh conditions in Procedure 1 using a high temperature and long cooking time leading to extensive cross-linking of sorghum proteins (Zhao et al., 2008).

There was no interaction (p = 0.60) between sample and procedure (i.e., the effect of SSM procedures on CHS was independent of tested samples). The primary important factor influencing CHS was amyloglucosidase dosage (p < 0.0001). CHS values increased steadily and significantly as levels of amyloglucosidase increased (Table 5.3). In procedure 3, the two samples had no statistical difference in CHS at low levels of amyloglucosidase (p = 0.07) but became distinct at higher levels (p < 0.005). Similar to pH adjustment, we expected a high level of amyloglucosidase to increase the resolution of SSM in differentiating samples.
When examining data in each procedure separately (Table 5.3), standard errors for Procedures 1, 2, and 3 were 0.83, 0.47, and 0.40, respectively. Therefore, Procedure 3 was selected, and its parameters were optimized further.

**Optimization of SSM Parameters**

There was no significant difference (p = 0.88) in CHS between the two buffers at pH 3.5 and 4.2 (Figure 5.4), suggesting that any 2 M sodium acetate buffers with pH values between 3.5 and 4.2 could be used for pH adjustment. The effect of mash pH on CHS was independent of tested samples (i.e., no interaction between sample and pH, p = 0.34). The CHS values adjusted by the buffer at pH 4.5 were slightly lower than those adjusted by the buffers at pH 3.5 and 4.2 (p < 0.003). Using the buffer at pH 5.5, CHS dropped significantly. The buffer at pH 4.20 was selected in this work, but was not indicative of the actual pH of the liquefied mash. Checking the pH, the actual pH of the mash was 4.4-4.6 at room temperature after adding 50 μL of 2 M sodium acetate buffer at pH 4.20 to each microtube.

The dosage range of Liquozyme was 0.013-0.015% (mass of enzyme to mass of corn ‘as is’), equivalent to 3-6 μL of enzyme per 30 g of sample (Novozymes, 2004b). The dosage range of Spirizyme was 0.04-0.06% (mass of enzyme to mass of corn ‘as is’), equivalent to 8-16 μL of enzyme per 30 g of sample (Novozymes, 2004a). The enzyme dosages (2×10 μL of Liquozyme and 100 μL of α-amylase per 30 g of dry grains) used in the traditional fermentation procedure were determined through an orthogonal test design considering factors such as mashing properties of grains, conversion efficiency, repeatability (unpublished data). For development of SSM, enzyme dosages were anticipated to be as small as possible, and CHS was expected to be as high as possible within an acceptable period of time. As shown in Figure 5.5, there was little difference in CHS among the three dosages of α-amylase when amyloglucosidase dosage was low (15 μL of amyloglucosidase per 30 g of sample). At higher dosages of amyloglucosidase, CHS increased significantly with increased α-amylase dosages. The effects of both enzyme dosages on CHS were synergetic (i.e., an interaction between α-amylase and amyloglucosidase, p < 0.0001). A minimum dosage of α-amylase, equivalent to 10 μL of Liquozyme per 30 g of sample, was necessary. For some samples, especially sorghum hybrids with tannins, we observed some gelled particles in the bottom of some microtubes when α-amylase dosage was 5 μL of Liquozyme per 30 g of sample. To ensure all slurries were...
completely dispersed during liquefaction, α-amylase dosage was determined as 20 μL of liquozyme per 30 g of sample in an SSM procedure. The highest CHS (62.4%) was achieved with the highest dosages of α-amylase and amyloglucosidase, and 87.3% of the total starch had been completely hydrolyzed to glucose in the sorghum II sample (Figure 5.5).

There was no statistical difference (p = 0.29) in CHS between 90 and 120 min (Figure 5.6). Thus, 90 min of saccharification could be used in SSM. The effect of amyloglucosidase dosage on CHS was independent of saccharification time (i.e., no interaction between dosage and time, p = 0.77). Increasing amyloglucosidase dosage was still an effective way to improve CHS, but the difference in CHS between 200 and 150 μL of Spirizyme per 30 g of sample was significantly (p < 0.0001) lower than that between 150 and 100 μL of Spirizyme per 30 g of sample. Additional experimentation verified higher levels of amyloglucosidase (e.g., 250 μL of Spirizyme per 30 g of sample) did not increase CHS significantly (data not shown). The highest CHS was 66.9%, which was approaching the theoretical value (71.5%) of the total starch of the sorghum II sample (Figure 5.6).

The optimized SSM procedure (Figure 5.7) was used to investigate the relationship between CHS and ethanol yield. All diluted mashes had very similar profiles to the chromatogram of a mash dilution set at room temperature for 3 days (the one with a dotted line in Figure 5.1), except their glucose peaks differed mainly in the magnitude of height. There was little maltose or maltotriose left in the mashes. Calculated mass of glucose was not significantly different (p = 0.11) from that obtained through direct measurement after all of the mash in a microtube had been diluted to a known volume (data not shown). Using a mini shaking incubator in this procedure made it possible to analyze up to 50 microtubes in one test cycle, which would increase efficiency and reduce the cost of per-sample analysis.

**Predicting Ethanol Yield with the SSM Procedure**

In addition to the nine primary samples, nine additional sorghum varieties were tested to evaluate their ethanol yields from the traditional fermentation and SSF procedures and their CHS values using the SSM procedure. The SSF procedure was used to mimic fuel ethanol production in the dry-grind industry. For all 18 sorghum hybrids, their ethanol yields (% v/v) were 11.99-14.55 (13.35 on average) in traditional fermentation, and 12.38-14.77 (13.75 on average) in SSF. The ethanol yields in traditional fermentation were highly related to those in SSF ($R^2 = 0.97$, p <
Ethanol yield improved significantly using the SSF procedure ($p < 0.0001$). The relative increases in ethanol yield were 0.6-4.9% (3.0% on average). Because amylglucosidase and yeast were added simultaneously, a concentrated glucose solution was avoided and the initial osmotic stress of yeast was lowered, which could be one reason why ethanol yield increased in the SSF procedure (Bothast and Schlicher, 2005). Another reason could be that the active dry yeast of an industry strain, Ethanol Red, was used in SSF.

With all 18 sorghum hybrids in 3 replications, the optimized SSM procedure had an overall standard error of 0.28. A majority of the starch had been hydrolyzed to glucose in the saccharified mash. The ratios of CHS to total starch ranged from 91.2% to 97.5% (94.1% on average). As shown in Table 5.4, total starch was highly correlated to CHS ($p < 0.0001$), but it explained only 82% of the variability in CHS.

As expected, total starch was correlated with ethanol yields ($R^2 = 0.78$, $p < 0.0001$ for traditional fermentation and $R^2 = 0.86$, $p < 0.0001$ for SSF). Compared with total starch, CHS was more powerful at predicting ethanol yield (Table 5.4). There were strong, linear relationships between CHS and ethanol yields in both fermentation procedures ($R^2 = 0.86$, $p < 0.0001$ for traditional fermentation and $R^2 = 0.93$, $p < 0.0001$ for SSF). Relationships between total starch, CHS, and ethanol yields in the SSF procedure were stronger than those in traditional fermentation. Results of multiple regression showed that the role of CHS was dominant ($p = 0.007$ in traditional fermentation and $p = 0.0003$ in SSF), even when combined with total starch to predict ethanol yield.

**CONCLUSIONS**

This research investigated the feasibility of using SSM as a method for predicting ethanol yield of sorghum hybrids and developed an SSM procedure with optimum parameters. This procedure had advantages including small quantity requirement for grain samples, use of common industry enzymes, high repeatability, high efficiency, and low cost of per-sample analysis. The 18 sorghum hybrids tested showed strong, linear correlations between CHS from SSM and ethanol yields from both traditional and SSF procedures. CHS proved a reliable indicator for ethanol yield.

**ACKNOWLEDGMENTS**
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REFERENCES


Figure 5.1 Chromatograms of a mash analyzed right after diluting by distilled water (solid line) and re-analyzed after 3 days at room temperature for the same dilution (dotted line). Liquefaction condition: 95°C, 60 min and 80°C, 30 min; 2×10 µL of Liquozyme per 30 g of sample. Saccharification condition: 60°C, 30 min; 100 µL of Spirizyme per 30 g of sample.
Figure 5.2 Inactivation of amyloglucosidase in diluted mashes from SSM. The same liquefaction and saccharification conditions as used for the SSM in Table 5.2. Treatment I, no inactivation (i.e., diluted by distilled water); treatment II, inactivation by boiling the mash dilution at 100°C for 10 min; treatment III, inactivation by 0.01 M phosphate buffer at pH 10.0. The numbers 0, 1, 2, and 3 following each treatment mean that the mash dilutions were set at room temperature for 0, 1, 2, and 3 days, respectively. Duplication for each combination of sample and treatment with standard error of 0.40 in the first experimental design.
Figure 5.3 Effect of pH adjustment on SSM. The same liquefaction and saccharification conditions as used for the SSM in Table 5.2, but no amyloglucosidase added in treatment I (i.e., distilled water as a substitute). Only in treatment III, pH adjusted after liquefaction by 50 μL of 2 M acetate buffer at pH 4.2. Amyloglucosidase was inactivated by 0.01 M phosphate buffer at pH 10.0. Duplication for each combination of sample and treatment with standard error of 0.39 in the second experimental design.
Figure 5.4 Optimization of pH for saccharification in SSM. Liquefaction condition: 86°C, 90 min; 20 µL of Liquozyme per 30 g of sample. Saccharification condition: 68°C, 90 min; 100 µL of Spirizyme per 30 g of sample. The pH of liquefied mashes was adjusted by 50 µL of 2 M acetate buffers at pH 3.5, 4.2, 4.5, and 5.5, respectively. Amyloglucosidase was inactivated by 0.01 M phosphate buffer at pH 10.0. Different superscript letters after pH values indicate significant differences (p < 0.05) among pH adjustments. Duplication for each combination of sample and buffer with standard error of 0.66 in the third experimental design.
Figure 5.5 Effect of enzyme dosages on CHS of a sample, sorghum II, in SSM. Liquefaction condition: 86°C, 90 min. Saccharification condition: 68°C, 90 min. The pH of liquefied mashes was adjusted by 50 μL of 2 M acetate buffer at pH 4.2. Amyloglucosidase was inactivated by 0.01 M phosphate buffer at pH 10.0. Duplication for each combination of enzyme dosages with standard error of 0.44 in the fifth experimental design.
Figure 5.6 Effect of amyloglucosidase dosage and saccharification time on CHS of a sample, sorghum II, in SSM. Liquefaction condition: 86°C, 90 min; 20 µL of Liquozyme per 30 g of sample. Saccharification condition: 68°C, 60-120 min. The pH of liquefied mashes was adjusted by 50 µL of 2 M acetate buffer at pH 4.2. Amyloglucosidase was inactivated by 0.01 M phosphate buffer at pH 10.0. Duplication for each combination of dosage and time with standard error of 0.46 in the sixth experimental design.
Figure 5.7 The schematic flow diagram of the SSM procedure with optimum parameters.
<table>
<thead>
<tr>
<th>design no.</th>
<th>design structure</th>
<th>factors and treatment structure (one-way or two-way factorial)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>whole-plot factors: 2 samples (Sorghum II and VIII); one-way.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sub-plot factors: 7 treatments (I-0, I-3, II-3, III-0, III-1, III-2, and III-3); one-way.</td>
</tr>
<tr>
<td>2</td>
<td>split-plot</td>
<td>whole-plot factors: 2 samples (Sorghum I and VII); one-way.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sub-plot factors: 3 treatments (I, II, and III); one-way.</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>whole-plot factors: 2 samples (Sorghum II and VIII); one-way.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sub-plot factors: 4 acetate buffers (pH 3.5, 4.2, 4.5, and 5.5); one-way.</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>whole-plot factors: 3 SSM procedures (1, 2, and 3); one-way.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sub-plot factors: 3 amylglucosidase dosages (15, 50, and 50 µL) and 2 samples (Sorghum II and VIII); two-way.</td>
</tr>
<tr>
<td>5</td>
<td>completely random</td>
<td>3 amylglucosidase dosages (15, 50, and 100 µL) and 3 α-amylase dosages (5, 10, and 20 µL); two-way.</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>3 amylglucosidase dosages (100, 150, and 200 µL) and 3 saccharification times (60, 90, and 120 min); two-way.</td>
</tr>
</tbody>
</table>

*Results in the first, second, third, fourth, fifth, and sixth experimental designs were shown in Figure 5.2, Figure 5.3, Figure 5.4, Table 5.3, Figure 5.5, and Figure 5.6, respectively.*
<table>
<thead>
<tr>
<th>sample code</th>
<th>total starch (%, db)</th>
<th>CHS (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>laboratory fermentation&lt;sup&gt;b,c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SSM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>analyzed by HPLC</td>
</tr>
<tr>
<td>I</td>
<td>70.4 b,c&lt;sup&gt;d&lt;/sup&gt;</td>
<td>37.8 c</td>
<td>40.0 b,c</td>
</tr>
<tr>
<td>II</td>
<td>71.5 a,b</td>
<td>35.8 d</td>
<td>38.6 c</td>
</tr>
<tr>
<td>III</td>
<td>68.0 d</td>
<td>37.5 c</td>
<td>41.3 b</td>
</tr>
<tr>
<td>IV</td>
<td>72.0 a,b</td>
<td>41.6 a</td>
<td>44.8 a</td>
</tr>
<tr>
<td>V</td>
<td>68.7 d</td>
<td>38.8 b,c</td>
<td>40.8 b,c</td>
</tr>
<tr>
<td>VI</td>
<td>71.1 b</td>
<td>41.7 a</td>
<td>40.8 b,c</td>
</tr>
<tr>
<td>VII</td>
<td>71.3 a,b</td>
<td>39.1 b,c</td>
<td>44.6 a</td>
</tr>
<tr>
<td>VIII</td>
<td>73.0 a</td>
<td>39.6 b</td>
<td>45.8 a</td>
</tr>
<tr>
<td>IX</td>
<td>68.8 c,d</td>
<td>34.3 d</td>
<td>35.2 d</td>
</tr>
</tbody>
</table>

replications 2 2 2 2
standard error 0.53 0.50 0.43 0.42
LSD (0.05) 1.69 1.61 1.38 1.33

<sup>a</sup> Liquefaction condition: 95°C, 60 min and 80°C, 30 min; 2×10 µL of Liquozyme per 30 g of sample. Saccharification condition: 60°C, 45 min; 100 µL of Spirizyme per 30 g of sample.

<sup>b</sup> Liquefaction at 95°C for 45 min and 80°C for 30 min; saccharification at 60°C for 30 min;

<sup>c</sup> Right after mashing, the liquid and solid parts of mashes were cooled, frozen, and lyophilized.

<sup>d</sup> Means followed by different letters in the same column are significantly different (p < 0.05).
Table 5.3 Effects of SSM Procedures and Amyloglucosidase Dosages on CHS (% db) of Mashes\textsuperscript{a}

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Amyloglucosidase dosage (µL of Spirizyme per 30 g sample)</th>
<th>SSM procedure\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>\textsuperscript{1}\textsuperscript{Ad}</td>
</tr>
<tr>
<td>II a\textsuperscript{c}</td>
<td>15</td>
<td>34.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>54.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>62.1</td>
</tr>
<tr>
<td>VIII b</td>
<td>15</td>
<td>37.0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>57.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>66.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Duplication for each combination of sample, dosage, and procedure with standard error of 0.60 in the fourth experimental design.

\textsuperscript{b} Procedure 1, liquefaction at 95°C for 90 min; Procedure 2, liquefaction at 95°C for 45 min, and at 86°C for 45 min; Procedure 3, liquefaction at 86°C for 90 min. For all procedures, the pH of liquefied mashes was adjusted by 50 µL of 2 M acetate buffer at pH 4.2, saccharification at 68 °C for 90 min, and the dosages of heat-stable α-amylase equivalent to 20 µL of Liquozyme per 30 g of sample. Amyloglucosidase was inactivated by 0.01 M phosphate buffer at pH 10.0.

\textsuperscript{c} Samples are significantly different with their codes followed by different letters in the first column (p < 0.05).

\textsuperscript{d} Procedures are significantly different with their codes followed by different capitals in superscript in the second row (p < 0.05).

\textsuperscript{e} Means followed by different letters in the last column are significantly different (p < 0.05).
<table>
<thead>
<tr>
<th>parameters</th>
<th>ethanol yield (%)&lt;sub&gt;a&lt;/sub&gt;</th>
<th>total starch (%)&lt;sub&gt;b&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>traditional fermentation</td>
<td>SSF</td>
</tr>
<tr>
<td>total starch (%)&lt;sub&gt;db&lt;/sub&gt;</td>
<td>0.78***</td>
<td>0.86***</td>
</tr>
<tr>
<td>CHS (%)&lt;sub&gt;db&lt;/sub&gt;c</td>
<td>0.86***</td>
<td>0.93***</td>
</tr>
</tbody>
</table>

*** Significant at 0.1% level.

<sup>a</sup> Liquefaction at 95°C for 45 min, and at 80°C for 30 min; saccharification at 60°C for 30 min.

<sup>b</sup> Liquefaction at 86°C for 90 min, no saccharification before fermentation.

<sup>c</sup> For SSM, the optimum liquefaction and saccharification conditions described in Figure 5.7.
CHAPTER 6 - CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Sorghum proteins are known to cross-link during cooking which reduces their digestibility. In this study, protein cross-linking occurring during the mashing step in the ethanol production process was investigated to determine its impact on ethanol production. Protein digestibility, solubility, and microstructures were characterized to provide insight into the amount of protein cross-linking occurring during the mashing process. Mashing was found to reduce protein digestibility to a greater degree than under conditions typically used to cook some sorghum foods. CFLSM images showed the presence of web-like protein structures, which could trap oligosaccharides, polysaccharides, or starch granules and reduce their availability to enzymes during ethanol production. Protein cross-linking was considered to be the main change during mashing and the degree of protein cross-linking varied among sorghum cultivars. As a marker of cross-linking, protein digestibility of the original samples was highly related to conversion efficiency. Solubility of proteins extracted from mashed sorghum, using an alkaline SDS borate buffer with extraction time of 24 hr, decreased substantially after mashing. Ethanol yield increased and conversion efficiency improved notably with the increase of extracted proteins from mashed samples. Both solubility and SE-HPLC peak area of proteins extracted from mashed samples were highly correlated with ethanol fermentation.

After encouraging results, a rapid method for extracting proteins from sorghum using sonication in conjunction with the SDS borate buffer was developed. From the mashed samples, a 30-sec sonication treatment extracted more protein than buffer-only, 24-hr extraction. In addition, sonication extracted more polymeric proteins from both the mashed and non-mashed samples compared with the buffer-only extraction method. CFLSM images showed that the web-like protein microstructures were disrupted during sonication. There were strong relationships between extractable proteins and fermentation parameters. Ethanol yield increased and conversion efficiency improved significantly as the amount of extractable proteins from sonication of mashed samples increased. The amount of proteins extracted using sonication and quantified by SE-HPLC indirectly reflected protein structures, which can determine the access of enzymes to sorghum starch and thus could be related to fermentation quality of sorghum. The
higher the degree of protein cross-linking, the less the amount of total protein and polymeric proteins in the sonication extract. In another words, the lower the degree of protein cross-linking, the easier the enzyme access to starch granules, gelatinized starch, or oligosaccharides held by the cross-linked protein matrix, and the better the fermentation performance.

The feasibility of using RVA for assessing fermentation quality of grain sorghum was extensively investigated in this study. For the 23-min gelatinization, pasting, and setback profile, there was a strongly linear relationship between ethanol yield and final viscosity as well as setback. RVA could be used for prediction of ethanol yield. Sorghum cultivars with higher peak and final viscosities, resulting from larger amounts of gelatinized substrates, produced higher ethanol yields than those with low viscosities. Differences in mashing properties among sorghum grains were enlarged and quantified using the 10-min liquefaction test. There was a remarkable difference in mashing properties among representative grains with the normal dosage of α-amylase. Tannin content was highly correlated to mashing properties. The 10-min RVA procedure could be used for measurement of tannin levels in sorghum grains. With the 10-min liquefaction profile, final viscosities decreased remarkably with increasing levels of heat-stable α-amylase in the slurries. There were clear power correlations between final viscosities and α-amylase levels. Different grains fitted different power curves, and the optimized enzyme doses varied greatly among grains to obtain less than 120 cP of final viscosities. RVA could be used for optimization of α-amylase doses and for assessment of different commercial enzyme preparations used in the fuel ethanol industry.

A small-scale mashing (SSM) procedure for predicting ethanol yield of sorghum grains was also developed in this study. This procedure was similar to the mashing step in a fermentation test, but only required a few hundred milligrams of test samples. The industry-used enzymes, including heat-stable α-amylase and amyloglucosidase, were employed in SSM as in fermentation procedures. Parameters such as temperature, pH, enzyme dosage, and saccharification time, were optimized, resulting in high repeatability, high efficiency, and low-cost per-sample analysis, etc. There were strong linear correlations between completely hydrolyzed starch (CHS) from SSM and ethanol yields from both traditional and simultaneous saccharification and fermentation procedures. CHS was a better indicator for predicting ethanol yield in fermentation than total starch.

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RECOMMENDATIONS

The role of disulphide crosslinking has been demonstrated in various *in vitro* studies. Protein cross-linking, mainly through disulphide bonding, appears to be perhaps the most important factor affecting cooked sorghum protein digestibility. In this study, mashing caused large decreases in protein digestibility and solubility. A reducing agent (β-ME) did help to extract more proteins from mashed residual sorghum pellets after preextraction with the SDS borate buffer for 24 hr or using sonication for 30 sec, which demonstrated that some polymers cross-linked by disulfide bonds developed during mashing. However, β-ME could not completely solubilize proteins in mashed sorghum. One possible explain for this is that the disulfide-mediated polymerization of kafirins upon mashing was so extensive that it retarded disulphide bonds accessible to β-ME or kafirins to pepsin, and resulted in low protein solubility and digestibility. Sonication is believed to reduce the molecular weight of protein complexes by breaking covalent bonds, thus rendering them soluble. In this study, for proteins extracted from mashed sorghum using sonication and separated by SE-HPLC, the area of fraction I decreased significantly after reduction by 2% β-ME. It is obvious that high-$M_w$ polypeptides decreased, while low-$M_w$ polypeptides increased, thus indicating some of the proteins were linked by disulphide bonds. However, fraction I did not disappear completely after adding β-ME, indicating that some proteins could not be degraded by this reducing agent. Those protein molecules that still appeared at fraction I were taken as non-disulphide cross-linked polymers, or at the minimum resistant to reducing agents. Thus, there might be non-disulphide cross-links existing in sorghum proteins such as dityrosine bridges, and this could be investigated in mashed sorghum.

The small-scale mashing (SSM) procedure worked well on sorghum grains. It is necessary to extend its utilization in other grains including corn, wheat, millet, rice, teff, etc. to predict ethanol yield. There is also a need to investigate the method for rapid glucose determination, with which the SSM procedure would make prediction of ethanol yield more efficient and accurate than total starch assay.