JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Impact of Mashing on Sorghum Proteins and Its Relationship to Ethanol Fermentation

RENYONG ZHAO,[†] SCOTT R. BEAN,*[,‡] BRIAN P. IOERGER,[‡] DONGHAI WANG,[†] AND DANIEL L. BOYLE[§]

Department of Biological and Agricultural Engineering, and Division of Biology, Microscopy Facility, Kansas State University, Manhattan, Kansas 66506, and Grain Marketing and Production Research Center, Agricultural Research Service, United States Department of Agriculture, Manhattan, Kansas 66502

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 because of cross-linking reduced conversion efficiency.

KEYWORDS: Sorghum; protein; kafirin; digestibility; solubility; 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 bioindustrial 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 to other cereals (*6, 7*), presumably through the 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

* To whom correspondence should be addressed. Telephone: (785) 776-2725. Fax: (785) 776-2792. E-mail: scott.bean@gmprc.ksu.edu.
[†] Department of Biological and Agricultural Engineering, Kansas State University.
[‡] United States Department of Agriculture.
[§] Division of Biology, Microscopy Facility, Kansas State University.

10.1021/jf072590r CCC: $40.75
Published on Web 01/16/2008

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 the 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 because of the conformation of the proteins not allowing reducing agents easy access to disulfide bonds (*14, 15*). The possibility of formation of nondisulfide 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 remainder 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 amyloglucosidase at 60 °C for 30 min. After cooling to room temperature, all of 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 Crop., 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 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 centrifugation at 9050*g* 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 1:10 000 (Sigma P-7000; activity 924 units per mg of protein). Cooked materials were prepared in two ways. One method (cooking 1) 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 (cooking 2) 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 13200*g* 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 13200*g* 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

Table 1. *In Vitro* Protein Digestibility and Fermentation Parameters

	protein digestibility (%)				fermentation parameters	
variety code	original[A a]	mashing[B]	cooking 1[C b]	cooking 2[D b]	ethanol yield (%, v/v)	conversion efficiency (%)
I	54.1 d[c]	21.7 b	31.0 a	27.4 a	13.17 e	86.3 d
II	48.4 e	18.3 c,d	23.0 b, c	22.0 c, d	13.13 e	84.7 e
III	23.0 g	0.6 e	0.6 d	0.5 e	12.36 f	83.9 f
IV	64.1 b	22.0 b	23.7 b, c	21.0 c, d	13.49 c	86.4 d
V	33.6 f	0.0 e	2.0 d	2.4 e	12.49 f	83.9 f
VI	63.9 b	16.4 d	22.6 c	20.2 d	13.50 c	87.6 c
VII	58.4 c	19.1 c	23.0 b, c	20.3 c, d	14.07 b	91.0 a
VIII	58.9 c	19.5 c	26.0 b	23.5 c, b	14.41 a	91.1 a
IX	68.2 a	25.3 a	25.9 b	25.3 a, b	13.33 d	89.4 b
replications	4	4	2	2	2	2
standard error	0.64	0.72	0.97	1.03	0.04	0.27
LSD (0.05)	1.85	2.08	3.10	3.30	0.13	0.85

[a] Capitals in superscript in the second row mean significantly different ($p < 0.05$) among the treatments. [b] 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. [c] Values followed by the same letter in the same column are not significantly different ($p < 0.05$).

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 were 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 the 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. The 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. To make data from different samples with different protein contents comparable, area per milligrams 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 H_2O 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 13200*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). Prior to imaging, one drop of 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 (*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 H_2O. 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 H_2O for 6 times to remove soluble sugars. In some experiments, proteins in mashed samples (0.5 g) were first extracted by different buffers (ratios of solvent/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 H_2O 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 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 ($R^2 = 0.99$, linear regression using the data in **Table 1**).

Impact of Mashing on Sorghum Protein Solubility. As shown in **Table 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

Table 2. Protein Solubility in Borate Buffer with or without Reducing Agent Added

	protein solubility (%)			
	original sorghum		mashed sorghum	
variety code	extraction with buffer 1[a]	sequential extraction with buffer 1 and 2[a]	extraction with buffer 1	sequential extraction with buffer 1 and 2
I	83.9 d[b]	95.1 a	19.8 d	66.3 e
II	89.1 b	95.4 a	20.3 d	68.6 d
III	77.2 e	93.3 b	8.3 f	41.2 g
IV	92.3 a	95.8 a	31.1 a	77.1 b
V	81.5 e	93.6 b	7.8 f	36.8 h
VI	85.6 c	93.2 b	16.7 e	60.3 f
VII	92.6 a	95.2 a	27.6 b, c	71.6 c
VIII	85.5 c	92.5 b, c	27.1 c	78.6 b
IX	89.6 b	92.0 c	30.0 a, b	83.9 a
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

[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] Values followed by the same letter in the same column are not significantly different ($p < 0.05$).

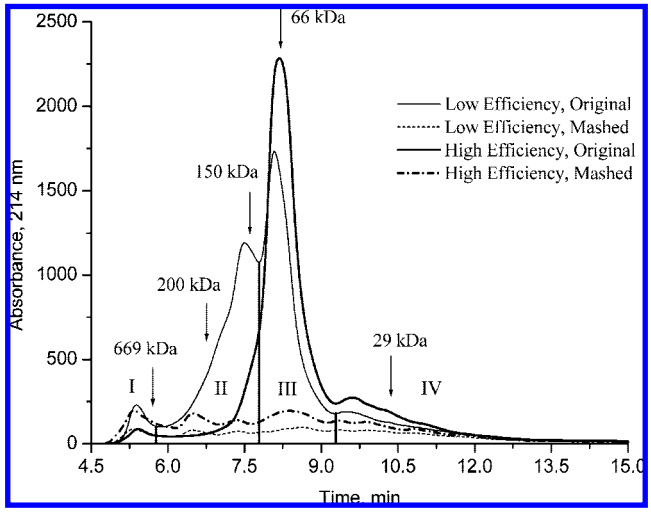

Figure 1. Typical SE-HPLC separations of proteins extracted with buffer 1 (12.5 mM sodium borate at pH 10.0 containing 2% SDS) from original and mashed sorghum samples with different conversion efficiencies.

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 nonreducing 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 1**. For comparative purposes, chromatograms were divided into four regions (indicated as I, II, III, and IV in **Figure 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

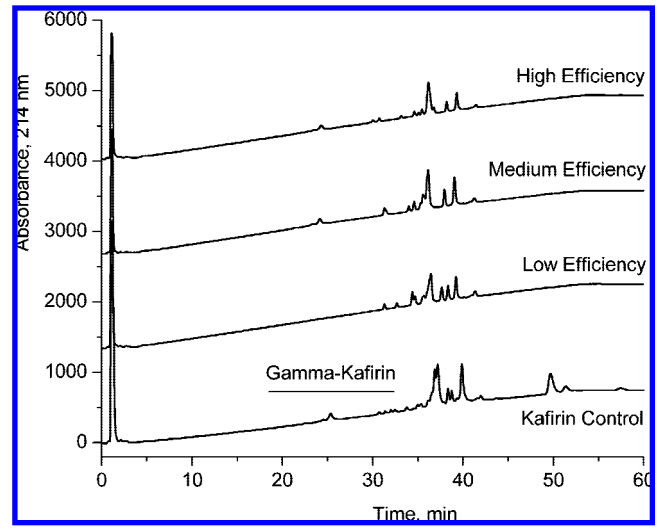

Figure 2. RP-HPLC separations of kafirins extracted from unmashed sorghum samples with different conversion efficiencies.

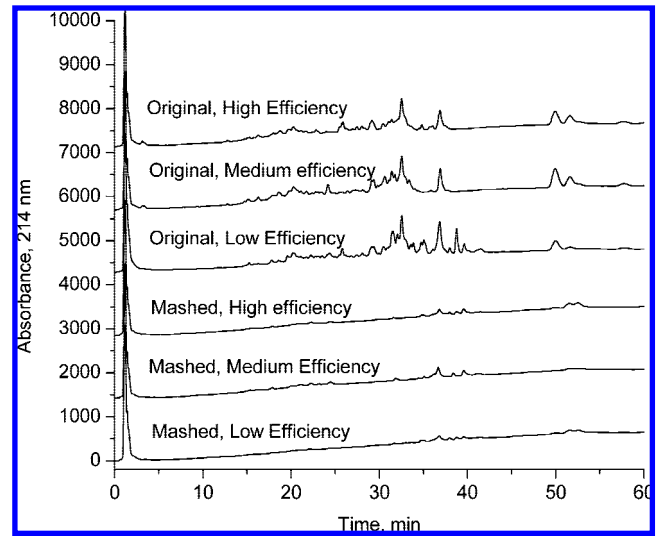

Figure 3. RP-HPLC separations of proteins extracted with buffer 2 (12.5 mM sodium borate at pH 10.0 containing 2% SDS and 2% β-ME) for 30 min twice from unmashed and mashed sorghum samples with different conversion efficiencies.

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**. 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 shows the RP-HPLC separations of kafirins extracted from unmashed cultivars that vary in conversion efficiency. γ-Kafirin (%) was calculated as a percentage of the area of γ-kafirin fraction to total area (including α, β, and γ fractions) in RP-HPLC chromatograms. γ-Kafirins (%) ranged from 0.33 to 7.61% (3.97% in average), with the remainder of the kafirins being α and β. **Figure 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 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

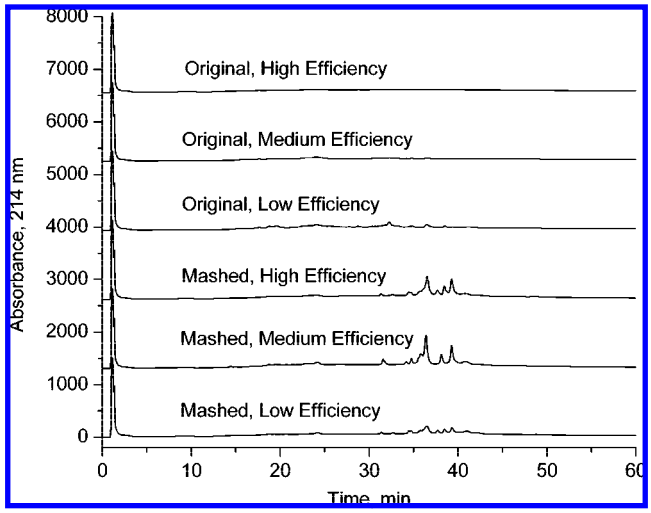

Figure 4. RP-HPLC separations of proteins extracted with buffer 2 (12.5 mM sodium borate at pH 10.0 containing 2% SDS and 2% β-ME) after extraction with buffer 1 (12.5 mM sodium borate at pH 10.0 containing 2% SDS) for 24 h from unmashed and mashed sorghum samples with different conversion efficiencies.

and thus more protein cross-linking would be expected because of protein−tannin interactions. **Figure 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 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 (parts **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 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 milligrams 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 3**).

Because of 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

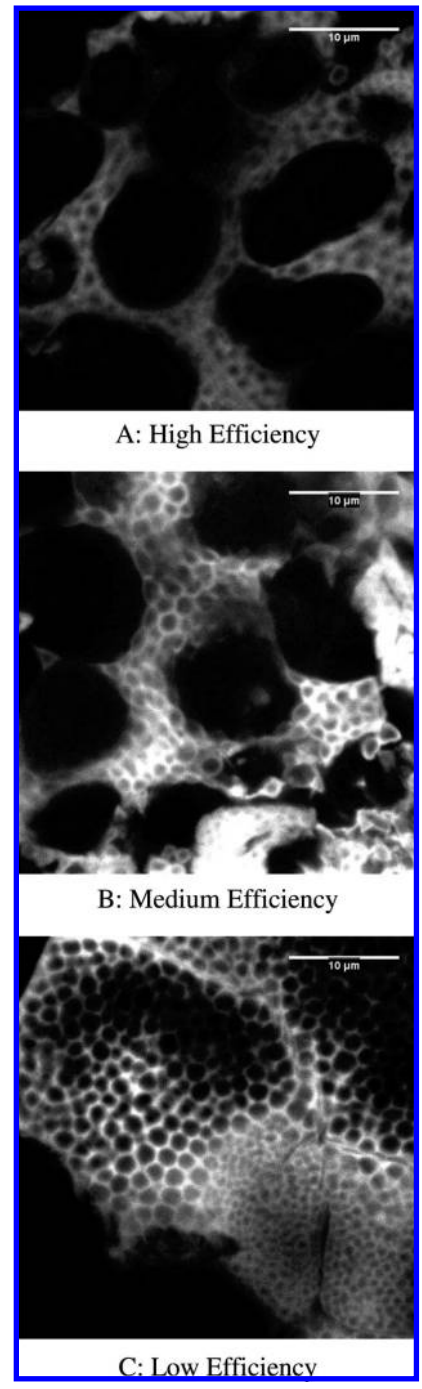

Figure 5. CFSLM images (single optical planes) of mashed sorghum samples with different conversion efficiencies, with the protein matrix (white-gray areas) stained with FITC.

starch had been completely hydrolyzed to glucose in the mashed samples (**Table 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). After

Table 3. Coefficient of Determination (R^2) for Solubility, SE-HPLC Area of Proteins from Mashed Sorgum, and Ethanol Fermentation

	protein solubility		SE-HPLC area/mg of protein	
ethanol fermentation	extraction with buffer 1[a]	sequential extraction with buffer 1 and 2	total area	area of fraction I
ethanol yield	0.604[b]	0.597[b]	0.807[d]	0.662[c]
conversion efficiency	0.545[b]	0.532[b]	0.735[c]	0.596[b]

[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 a 5% level. [c] Significant at a 1% level. [d] Significant at a 0.1% level.

the standard procedure for total starch assay of samples containing glucose and maltosaccharides (*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 4**).

DISCUSSION

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 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 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 M_r values, such as fraction I in **Figure 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** 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**, 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 3** and **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 nondisulphide cross-links existing in sorghum proteins after mashing (*18*).

Table 4. Glucose and Total Starch Analyses in Mashed Sorghum and Various Residual Pellets[a]

		total starch in various residues (%)			
		mashed sample		pellets after protein extraction	
variety code	glucose in mashed sorghum (%)	washing with 80% ethanol	washing with water[Ab]	extraction with buffer 1[B]	sequential extraction with buffer 1 and 2[C]
I	55.4 e[c]	12.0 f	2.6 e	1.3 e	0.9 e
II	52.9 f	14.8 d	3.1 c	1.6 c	0.9 e
III	60.6 a, b	17.9 a	3.7 a	2.0 a	1.3 b
IV	59.5 b, c	9.5 h	1.6 g	0.8 f	0.5 f
V	58.6 c, d	17.5 b	3.0 d	2.0 a	1.3 a
VI	58.0 d	11.9 f	2.0 f	1.5 d	1.2 c
VII	61.0 a	10.0 g	1.5 h	0.7 g	0.4 g
VIII	60.4 a, b	13.8 e	3.2 b	1.7 b	1.2 d
IX	49.6 g	16.5 c	1.3 i	0.3 h	0.2 h
replications	2	2	2	2	2
standard error	0.44	0.08	0.02	0.01	0.01
LSD (0.05)	1.40	0.27	0.06	0.03	0.02

[a] All values were calculated as a percentage of glucose or residual starch content to total starch content. [b] Capitals in superscript in the third row mean significantly different ($p < 0.05$) among the treatments. [c] Values followed by the same letter in the same column are not significantly different ($p < 0.05$).

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 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 milligrams of protein and ethanol yield, as well as conversion efficiency (**Table 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 ungelatinized 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 used 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 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

ACN, acetonitrile; β-ME (2-ME), β-mercaptoethanol; CFLSM, confocal laser-scanning microscopy; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetraacetic acid; FITC, fluorescein isothiocyanate; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; SE-HPLC, size-exclusion high-performance liquid chromatography; TFA, trifluoroacetic acid; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride.

ACKNOWLEDGMENT

We thank Novozymes, Inc. for providing Liquozyme SC DS and Spirizyme Fuel used in this research.

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Received for review August 30, 2007. Revised manuscript received November 28, 2007. Accepted December 10, 2007. This project was supported by the National Research Initiative of the USDA Cooperative State Research, Education, and Extension Service, Grant 2004-35504-14808. The U.S. Department of Agriculture, Agricultural Research Service, Northern Plains Area, is an equal opportunity/affirmative action employer, and all agency services are available without discrimination. The mention of firm names or trade products does not constitute endorsement by the U.S. Department of Agriculture over others not mentioned.

JF072590R