

Interaction Between Sorghum Protein Extraction and Precipitation Conditions on Yield, Purity, and Composition of Purified Protein Fractions

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ABSTRACT

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Sorghum proteins have the potential to be used as a bio-industrial renewable resource for applications such as biodegradable films and packaging. This project was designed to evaluate the effect of interactions between sorghum protein extraction and precipitation conditions on the yield, purity, and composition of sorghum protein fractions. Proteins were extracted with 70% ethanol under nonreducing conditions, with ultrasound, or under reducing conditions using either sodium metabisulfite or glutathione as the reducing agent. Several conditions were used to isolate the extracted proteins through precipitation, including lowering ethanol concentrations alone or in combination with lowering to pH 2.5, or by adding 1M NaCl to the extract. Combinations of these conditions were also tested. All precipitation conditions effectively precipitated proteins

and lowering the pH and adding 1M NaCl to the extracts enhanced precipitation in some cases. However, the conditions that precipitated the maximum amount of protein or highest purity of protein varied according to how the proteins were initially extracted. Precipitated proteins were characterized by RP-HPLC, SEC, HPCE, and SDS-PAGE to compare the protein fractions composition. Nonreduced and sonicated samples had a much wider M_w distribution than reduced extracts. Thus, extraction and precipitation conditions influenced the isolated proteins yield, purity, and composition. Because the extraction and purification processes influenced the composition, purity, and biochemical properties, it may be possible to prepare protein fractions with unique functionalities for specific end-uses.

Production of bioplastics made from renewable resources offers potentially lower energy consumption and greenhouse effects. Furthermore, bioplastics can be made to degrade completely after use, significantly reducing solid waste disposal problems (Bastioli 2001; Fritz et al 2001). By using biodegradable materials in packaging, benefits may be realized ecologically as well as socially (Fritz et al 2001). Currently, bioplastics are used in such applications as composting bags, fast food tableware, packaging, and mulch, with the major bioplastics used made primarily from starch and polylactic acid (Bastioli 2001).

Agricultural proteins such as soy, maize, and wheat proteins offer another source for the production of bioplastics and biodegradable materials. Soy proteins have been used for the production of a wide number of products including adhesives (Sun 2001; Sun and Bian 2001; Liu and Li 2002), plywood (Hojilla-Evangelista 2002), biodegradable films (Kim et al 2002), and bioplastics (Mungara et al 2002). Wheat gluten proteins have been used to make films for edible biodegradable film coatings (Mangavel et al 2002), food containers, and nonfood industrial applications such as insect and weed control, glues, and detergents (Bietz and Lookhart 1996). Maize proteins are used in a wide number of applications such as fiber production, chewing gum, adhesives, coatings, inks, and biodegradable plastics (Shukla and Cheryan 2000; Lawton 2002). These examples demonstrate the technological possibility of using agricultural proteins as renewable resources where bio-products are utilized.

Maize proteins have been traditionally extracted with aqueous alcohols for industrial uses (Shukla and Cheryan 2001; Lawton 2002). Dickey et al (2001) reported on the use of 70% ethanol to extract maize proteins with subsequent purification of the proteins from co-extracted lipids by lowering the ethanol concentration to 60%. Maize proteins were then precipitated from solution by

lowering the ethanol concentration further to 50% (Dickey et al 2001). The maize protein extraction procedure of Dickey et al (2001) was used as a starting point in this research to investigate precipitating sorghum proteins from aqueous ethanol solutions. For sorghum proteins, aqueous 1-propanol or tertiary-butanol is often used to extract sorghum proteins, and extensive studies on the extraction and solubility of sorghum proteins have been conducted (Sastry and Virupaksha 1969; Beckwith 1972; Wall and Paulis 1978; Evans et al 1987; Shull et al 1991; Hamaker et al 1995; Bean et al 2000; Park and Bean 2003). It is possible that while other organic solvents or detergents may improve the extraction of sorghum proteins in terms of yield, the final selection of solvent used industrially would depend on cost, toxicity, disposal of waste solvent, etc., with aqueous ethanol being the most practical solvent (Erasmus and Taylor 2003).

Sorghum has potential for bio-industrial applications such as production of ethanol and lactic acid (Zhan et al 2003). Sorghum proteins have been used to produce biodegradable films (Buffo et al 1997; Taylor et al 2004; Emmambux et al 2004; da Silva and Taylor 2005) and sorghum has been used as an extender in plywood adhesives (Ramos et al 1984) and in low-cost adhesives, wallboard, and packaging materials (Rooney and Waniska 2000). Sorghum has been converted into activated carbon as well (Diao et al 2002). Sorghum proteins have been extracted from sorghum bran for commercial production (da Silva and Taylor 2004).

The preceding examples, plus the fact that sorghum is a relatively drought-resistant cereal, demonstrate that sorghum has potential to be a valuable renewable resource for bio-industrial products, especially in dry areas where other crops are not as easily grown (McLaren et al 2003).

Therefore, the objectives of this research were to 1) evaluate interactions between initial protein extraction conditions (under conditions relevant to industrial uses) and protein isolation conditions on protein recovery and purity, and 2) characterize and compare proteins extracted and purified under different conditions to determine whether specific groups of proteins could be purified by manipulating the extraction and isolation procedures.

MATERIALS AND METHODS

Sample Preparation

The sorghum hybrid NC+ 371, grown in 2001 in south central Kansas, was used for all tests. Whole sorghum grain was ground with a Udy mill (Udy Corp., Fort Collins, CO) with a 0.2-mm

¹ USDA-ARS, Grain Marketing and Production Research Center, Manhattan, KS 66502. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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screen and stored at -20°C until needed and was equilibrated to room temperature before extractions.

Protein Extraction

Proteins were extracted using a modified method of Dickey et al (2001). First, 100 g of whole ground sorghum flour was extracted with 350 mL of 70% ethanol (v/v) at 50°C for 1 hr. Samples were vortexed briefly every 15 min during extraction. In some experiments, reducing agents (89.7 mM sodium metabisulfite or 14.8 mM glutathione) were added to 70% ethanol (v/v) for the initial extraction of sorghum proteins. After centrifugation at 4,000 rpm for 10 min, the supernatant was decanted and diluted to 60% ethanol (v/v) to precipitate lipids from the extract and the sample was then centrifuged at 0°C for 30 min at 4,000 rpm (Dickey et al 2001). After centrifugation, the supernatant was used directly to isolate sorghum proteins through precipitation. For samples extracted with the aid of ultrasound, an ultrasonic processor was used (V1500, Sonics and Materials, Newton Town, CT). Samples were dispersed in 70% ethanol solution with mechanical stirring and then a probe was placed in the slurry for sonication. Sample containers were placed in an ice water bath to prevent heat buildup during sonication. Samples were sonicated for 4 min at an instrument setting of 80% using a 2.5-cm probe.

Protein Precipitation

Extracted samples were precipitated by lowering the ethanol concentration of the extract after removal of lipids (60%, v/v) from 50 to 30% (v/v) by dilution with water and in combination with addition of sodium chloride (1M) or by lowering to pH 2.5. To lower the pH, 50 mM sodium phosphate buffer, pH 2.5, was added to the samples and the mixture was vortexed briefly. Com-

binations of all these methods were also investigated. To precipitate the proteins, aliquots of the 60% ethanol (v/v) solution were placed into 50-mL centrifuge tubes and solvent conditions were manipulated as described previously. The samples were then vortexed and centrifuged at 0°C at 4,000 rpm for 10 min. Samples were decanted and precipitates were air-dried overnight at room temperature. Dried precipitates were weighed and analyzed for protein content by nitrogen combustion. Dried precipitates were also used directly for protein characterization. Solvent blanks (60% ethanol, v/v) were also carried through the precipitation procedures to check for precipitation of salts. The weight of precipitate was corrected by subtracting the weight of any salts that precipitated in the solvent blanks.

Protein Characterization

Reversed-phase high-performance liquid chromatography (RP-HPLC) separations were conducted using an HPLC system (1100, Agilent, Palo Alto, CA). RP-HPLC conditions were similar to those described in Bean et al (2000). Isolated protein fractions were redissolved in either 60% 1-propanol (v/v) +2% β -mercaptoethanol (β -ME) (Park and Bean 2003) or 60% 1-propanol (v/v) with no β -ME. Free-zone capillary electrophoresis (FZCE) was conducted using an HPCE system (MDQ, Beckman, Palo Alto, CA). FZCE conditions followed the procedures of Bean et al (2000). For FZCE, samples were redissolved in 60% 1-propanol (v/v) + 2% β -ME or 60% 1-propanol (v/v) with no β -ME (Park and Bean 2003). SDS-PAGE was conducted as described in (Bean 2003). For SDS-PAGE, samples were redissolved in SDS-PAGE sample buffer as described in Bean (2003) with or without 2% β -ME. Size-exclusion chromatography (SEC) was conducted using an Agilent 1100 HPLC system with a BioSep SEC-3000 column (Phenomenex, Torrance, CA). The mobile phase for SEC was a pH 7.0 sodium phosphate buffer (50 mM) with 1% SDS added. Column temperature was maintained at 30°C and flow rate was 0.5 mL/min. The samples for SEC analysis were redissolved in a pH 10.0 sodium borate buffer (12.5 mM) plus 1% SDS with or without 2% β -ME (Hamaker et al 1995). Standard proteins thyroglobulin (669 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), and carbonic anhydrase (29 kDa) were analyzed to estimate the molecular weight distribution of the sorghum proteins separated by SEC.

Nitrogen Combustion

Nitrogen content of sorghum flour and isolated protein fractions was determined by nitrogen combustion using a nitrogen determinator (FP-528, Leco, St. Joseph, MI) according to Approved Method 46-30 (crude protein-combustion method) (AACC International 2000). Nitrogen values were multiplied by 6.25 to convert to protein values.

Experimental Design and Statistical Analysis

Samples were analyzed in a randomized complete block design with two replicates for every treatment. Data were analyzed using the Proc ANOVA procedure with statistical analysis software (v. 8.2, SAS Institute, Cary, NC)

RESULTS

Initial Screening of Reducing Agents

For the initial screening of reducing agents, sorghum proteins were extracted with several reducing agents that could be used industrially and analyzed by RP-HPLC (Fig. 1). The reducing agents tested were sodium metabisulfite, glutathione, and cysteine. Reducing agents were used at the highest solubility levels possible in 70% ethanol (v/v). Note that this means reducing agents were not compared on equal molarities but on the maximum amount of reducing agent that could be dissolved in 70% ethanol. Both the type and concentration of reducing agents have influenced the

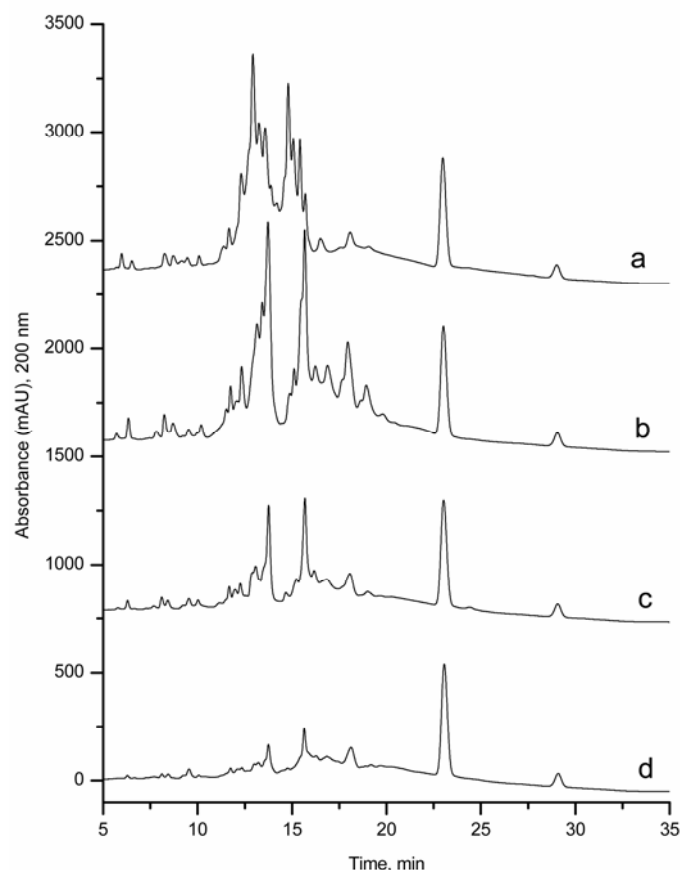


Fig. 1. RP-HPLC separations of kafirins extracted with 70% ethanol plus a) sodium metabisulfite, b) glutathione, c) cysteine, and d) no reducing agent (control).

extraction of sorghum proteins (Park and Bean 2003), which explains why reducing agents were used at maximum solubility possible. Based on the RP-HPLC profiles, both sodium metabisulfite and glutathione extracted higher amounts of protein than the other reducing agents. RP-HPLC chromatograms of samples extracted with these two reducing agents differed from each other slightly, suggesting that the reducing agent may be selectively extracting particular proteins. Therefore, both reducing agents were selected for further use in this study.

Amount of Total Protein Extracted

The total amount of protein extracted under reducing and non-reducing conditions was measured by analyzing the residue left after the initial extraction of the ground sorghum using nitrogen combustion. When 70% ethanol was used as the solvent, low levels of protein were extracted; <4% of the total protein was extracted. The use of 70% ethanol in combination with ultrasound increased the extraction to 10%. Protein extraction increased when sodium metabisulfite was used with 70% ethanol with ≈70% of the total protein extracted. Nitrogen combustion of the residue left after extracting with glutathione could not be conducted due to the nitrogen in the glutathione itself. However, RP-HPLC analysis and quantitation of the extracted proteins by UV showed that glutathione extracted amounts of protein similar to the sodium metabisulfite (data not shown).

Extraction and Precipitation of Sorghum Proteins

The amounts of sorghum protein obtained under the four extraction conditions tested in this project are listed in Table I. Data are

presented as the total amount of material precipitated (presented as mg/mL of 60% ethanol extract), the amount of protein precipitated (presented as mg/mL of 60% ethanol extract), as well as % protein of the precipitate.

For the nonreducing conditions, the highest total mass of precipitate as well as total mass of protein was recovered when the ethanol concentration of the protein extracts was lowered to 40% (v/v) in combination with the addition of 1M NaCl at pH 2.5 (Table I). However, the highest concentration of protein (purity) was present in the precipitates isolated by lowering ethanol to 50% (v/v) in the presence of 1M NaCl. The protein content of the precipitates ranged from 21 to 58%.

For proteins isolated after extraction with glutathione, the largest amount (mg) of precipitate was recovered when the ethanol concentration was lowered to 30% (v/v) in combination with 1M NaCl (Table I). Several precipitation conditions showed similar amounts of protein in the precipitates; the highest concentration in the precipitates occurred when the initial solvent was adjusted to either 40% ethanol or 40% ethanol (v/v) at pH 2.5. Protein concentrations of precipitates were 36–80% protein, which were much higher than those extracted under nonreducing conditions.

For proteins extracted with sodium metabisulfite, the greatest amount of precipitate (mg) was recovered when the ethanol concentration was decreased to 30% (v/v) with 1M NaCl (Table I). The highest concentration of protein (%) in the precipitates occurred when the ethanol concentrations were reduced to 30–50% or reduced to 50% ethanol (v/v) at pH 2.5. Protein concentrations of the precipitates were 50–86%, which were larger than precipitates prepared from the other extraction conditions.

TABLE I
Sorghum Protein Obtained Under Four Extraction Conditions

Precipitation Condition	70% Ethanol Only			70% Ethanol + Glutathione		
	Precipitate ^a (mg)	Protein ^a (mg)	Protein ^b (%)	Precipitate ^a (mg)	Protein ^a (mg)	Protein ^b (%)
40% Ethanol	0.25i	0.08g	30.7	5.93cd	4.72ab	79.5
50% Ethanol	1.45h	0.75f	51.7	7.52bc	5.21a	69.3
30% Ethanol/1M NaCl	3.71c	1.52b	41.0	16.67a	6.07a	36.4
40% Ethanol/1M NaCl	6.50b	1.36cd	20.9	9.70bc	5.41a	55.8
50% Ethanol/1M NaCl	2.39f	1.39cd	58.4	10.29	6.88a	66.8
30% Ethanol/pH 2.5	2.84d	1.39cd	48.8	3.29de	2.40bc	73.1
40% Ethanol/pH 2.5	2.58ef	1.31d	50.8	6.67b-d	5.32a	79.8
50% Ethanol/pH 2.5	1.89	0.98e	52.0	7.91bc	5.47a	69.2
30% Ethanol/1M NaCl/pH 2.5	3.60c	1.37cd	38.1	10.42b	4.92a	47.2
40% Ethanol/1M NaCl/pH 2.5	8.95a	2.76a	30.8	8.45bc	4.80a	56.8
50% Ethanol/1M NaCl/pH 2.5	2.80de	1.46bc	52.3	8.12bc	5.40a	66.5

^a Presented as mg/mL of 60% ethanol extract.

^b Presented as % protein of the precipitate.

^c Values followed by different letters in columns indicate significantly different means at $P < 0.05$.

TABLE I (continued)
Sorghum Protein Obtained Under Four Extraction Conditions

Precipitation Condition	70% Ethanol Only + Sodium Metabisulfite			70% Ethanol + Sonication		
	Precipitate ^a (mg)	Protein ^a (mg)	Protein ^b (%)	Precipitate ^a (mg)	Protein ^a (mg)	Protein ^b (%)
30% Ethanol	8.11cd	6.67a	82.2	0.7h	0.4b	54.1
40% Ethanol	7.83c-e	6.45ab	82.4	1.1h	0.6b	56.3
50% Ethanol	7.43de	6.28ab	84.5	4.2g	2.8a	66.7
30% Ethanol/1M NaCl	12.64a	6.37ab	50.4	9.0a	3.0a	33.7
40% Ethanol/1M NaCl	11.47b	6.12ab	53.4	8.0ab	2.7a	33.8
50% Ethanol/1M NaCl	6.96e	5.28c	75.8	5.3ef	3.0a	56.5
30% Ethanol/pH 2.5	8.36cd	6.57a	78.6	5.4ef	2.9a	54.1
40% Ethanol/pH 2.5	8.00c-e	6.56a	82.1	5.2e-g	2.9a	55.6
50% Ethanol/pH 2.5	7.32de	6.26ab	85.5	4.4fg	2.6a	58.9
30% Ethanol/1M NaCl/pH 2.5	11.69ab	5.88bc	50.3	7.3bc	3.0a	40.4
40% Ethanol/1M NaCl/pH 2.5	12.53ab	6.57a	52.4	6.7cd	2.6a	39.3
50% Ethanol/1M NaCl/pH 2.5	8.72c	6.10ab	69.9	6.0de	3.0a	50.5

^a Presented as mg/mL of 60% ethanol extract.

^b Presented as % protein of the precipitate.

^c Values followed by different letters in columns indicate significantly different means at $P < 0.05$.

For sonicated samples, the largest mass of precipitate was recovered when the ethanol concentration was reduced to 30% with 1M NaCl (Table I). Most of the precipitation conditions had similar amounts of protein in the precipitate. The highest amount of protein in the precipitate was recovered by lowering the ethanol to 50% (v/v), although lowering the ethanol to 50% (v/v) at pH 2.5 and lowering to 50% (v/v) with 1M NaCl showed similar levels. Protein content in the precipitates was 34–67%, which was higher than proteins extracted with 70% ethanol (v/v) alone.

Characterization of Isolated Sorghum Protein Fractions

Sorghum proteins extracted and isolated by the procedures used in this project were characterized by several analytical techniques to determine whether the composition of proteins isolated by different precipitation procedures varied. Isolated proteins were resuspended and analyzed by RP-HPLC, FZCE, SEC, and SDS-PAGE to determine whether isolated proteins varied in surface hydrophobicity, charge density, or M_w . Only minor differences occurred when samples were extracted under the same conditions but precipitated under different conditions. An example of this is shown in Fig. 2. Note that these chromatograms were normalized to the highest peak to account for differences in total protein of the isolates.

Very minor qualitative differences were noted for 15–17 min and minor quantitative differences were noted for 22–23 min. Therefore, for simplicity, one representative sample from each extraction group was selected for comparison of the protein com-

position between the various extraction conditions. Representative samples were selected from the precipitation conditions that resulted in high levels of protein in the precipitated fractions (high purity) that would likely be used in commercial applications.

To evaluate the M_w distributions of the isolated proteins, samples were analyzed by SEC and SDS-PAGE, both with and without added reducing agent. When samples were analyzed by SEC with no additional reducing agent added, large variations in M_w distribution were observed (Fig. 3A). The samples isolated from 70% ethanol extractions and those extracted with sonication had a much wider M_w range than the samples originally extracted with glutathione and sodium metabisulfite. Once the samples were reduced further with β -ME, the molecular distributions were very similar (Fig. 3B). The same results were present with SDS-PAGE (data not shown).

Samples were also separated by RP-HPLC and FZCE. Samples extracted with only 70% ethanol and 70% ethanol (v/v) in combination with sonication had much different patterns than those extracted with glutathione or sodium metabisulfite (Fig. 4A) until reduced, when they resembled the other samples in the RP-HPLC chromatograms (Fig. 4B). FZCE separations were similar (Fig. 5)

DISCUSSION

The endosperm proteins of sorghum consist mainly of kafirins, some of which are highly disulfide cross-linked (Oria et al 1995; El Nour et al 1998). It has long been known that reducing agents are necessary to efficiently extract sorghum proteins (Wall and Paulis 1978) and yields without using reducing agents can be low (Buffo et al 1997). In laboratory research, the reducing agents dithiothreitol and β -ME are often used in extracting sorghum proteins; however, these reducing agents are not suitable for industrial uses (Erasmus and Taylor 2003). Therefore, other reducing agents were used in this research. Sodium metabisulfite has been used in preparing sorghum foods to increase the protein digestibility (Hamaker et al 1987; El Khalifa et al 1999). Sodium metabisulfite has also been used in the extraction of maize zein (Carter and Reck 1970; Shukla and Cheryan 2001). Glutathione has been used as a reducing agent to investigate wheat flour rheology (Berland and Launay 1995). While sodium metabisulfite has been used previously to reduce sorghum proteins, to the best of our knowledge, glutathione has not.

The interactions between the types of reducing agent used in extracting sorghum proteins and the conditions used to isolate the proteins were also evaluated. Reducing agents vary in effectiveness in assisting with the extraction of sorghum proteins (Park and Bean 2003) and the data shown in Fig. 1 indicate that all reducing agents do not reduce sorghum proteins to the same degree. Therefore it is possible that the type of reducing agent used in the initial extraction of sorghum proteins will influence later isolation and purification steps. Note that the sodium metabisulfite solutions were acidic; at the concentration of the initial extraction they were pH 4.3 (measured in pure water without added ethanol). Thus this low pH could be one reason for the differences in the behavior between the samples extracted with sodium metabisulfite and the other samples.

Ultrasound has been used to improve the extraction of wheat proteins by breaking up the large polymeric proteins (Singh et al 1990; Bean and Lookhart 2001) presumably by breaking disulfide bonds (MacRitchie 1975). Ultrasound (sonication) has also been used to enhance the extraction of sorghum proteins (El Nour et al 1998). When ultrasound was used in combination with 70% ethanol to extract sorghum proteins, slight increases in the amount of protein extracted occurred as compared with nonreducing conditions. However, increases were not as high as expected. From research conducted on wheat proteins extracted during sonication, it was hypothesized that the ultrasonic energy would break disulfide bonds in the sorghum proteins and greatly enhance their solu-

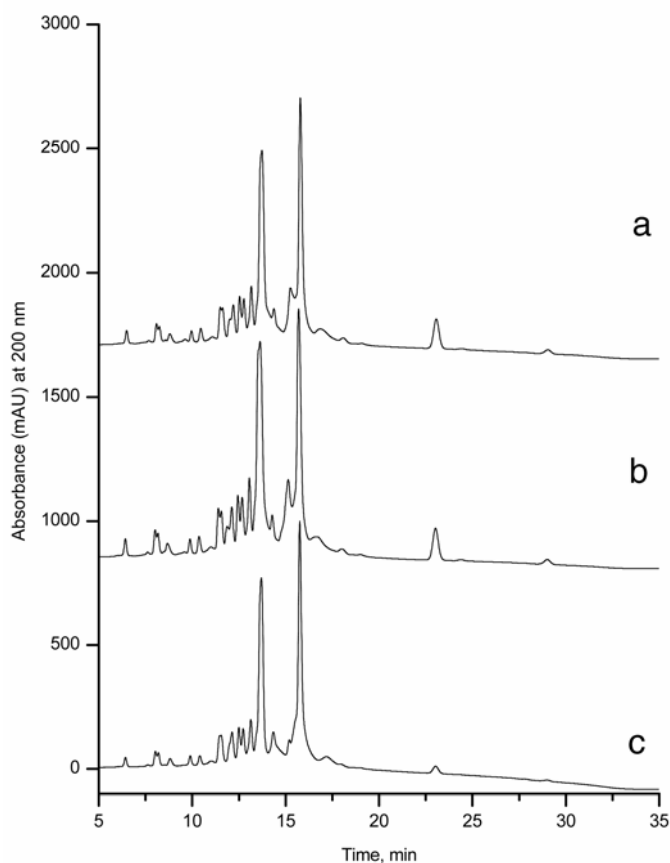


Fig. 2. RP-HPLC separations of kafirins initially extracted with 70% ethanol plus glutathione and precipitated by addition of 1M NaCl and lowering the ethanol concentration from 65% to a) 50%, b) 40%, and c) 30%. Patterns have been normalized to the highest peak (arbitrarily set at a value of 1) to account for quantitative differences and for easier qualitative comparison of the patterns. mAU values multiplied by 1,000 for scale similar to other figures.

bility. Further optimization of large-scale sonication as a method for large-scale sorghum protein extraction must be conducted.

Extracted proteins were effectively precipitated by all the conditions used in this study. Kafirins, the major endosperm protein of sorghum, require high levels of organic solvents, detergents, or chaotropes for extraction (Taylor and Schussler 1984; Hamaker et al 1995; Bean et al 1998). Kafirins often precipitate under conditions used to extract other cereal proteins (Taylor and Schussler 1984). Three different conditions were used to precipitate extracted proteins. The primary method used to precipitate the sorghum proteins was by lowering the ethanol content (Dickey et al 2001). Amino acid composition data (Lasztity 1995) and deduced amino acid sequences (Leite et al 1999) show that sorghum kafirins are characterized by high levels of glutamine, glutamic acid, leucine, proline, and alanine and along with maize proteins are considered to be the most hydrophobic cereal storage proteins. Thus by lowering the ethanol content the solvent becomes more aqueous (more polar) and protein solubility decreased.

In conjunction with lowering the ethanol content, the pH was lowered to 2.5 by the addition of a phosphate buffer to the extracts. Sorghum proteins have poor solubility at acidic pH (Taylor and Schussler 1984; Park and Bean 2003). Kafirins, the most abundant protein of sorghum, have low levels (<2%) of the positively charged amino acids arginine, lysine, and histidine (Lasztity 1995) that would carry charged side chains at acidic pH, which would play a role in the solubility of the proteins at low pH. Thus it is not surprising that lowering the pH would reduce the solubility of the sorghum proteins and enhance protein precipitation.

The use of NaCl to precipitate sorghum proteins along with lowering the ethanol concentration was also evaluated. Salts are known to have effects on protein stability and solubility (Timasheff and Arakawa 1989). NaCl is known to salt out or precipitate proteins (Arakawa and Timasheff 1984; Timasheff and Arakawa 1989). The major proteins of sorghum are the kafirins, which are prolamins. Prolamins typically, but not always, have poor solubility in water or salt solutions (Mifflin and Shewry 1974; Shewry et al 1994, 1996). Other cereal prolamins, for example wheat gluten, have low solubility in NaCl (Preston 1981). Thus it was expected that adding 1M NaCl to sorghum protein extracts would facilitate protein precipitation. In several instances, adding NaCl to the protein samples improved the precipitation over dilution of the ethanol concentration alone.

A combination of all three precipitation methods also was investigated, although there was no improvement in the amount of protein precipitated over the other conditions tested in this study.

The precipitation conditions used in this study, therefore, may affect sorghum protein solubility by changing the polarity of the initial extraction solvent as well as changing the charge density of the proteins. This points to the fact that precipitation conditions can be manipulated not only to vary the amount and purity of the proteins precipitated (Table I) but also the biochemical properties of the proteins (Figs. 3, 4, and 5).

In addition, the precipitation conditions that yielded the largest amount of precipitate and highest purity of precipitates varied according to how the proteins were extracted initially, including the two reducing agents used. Thus isolation procedures should

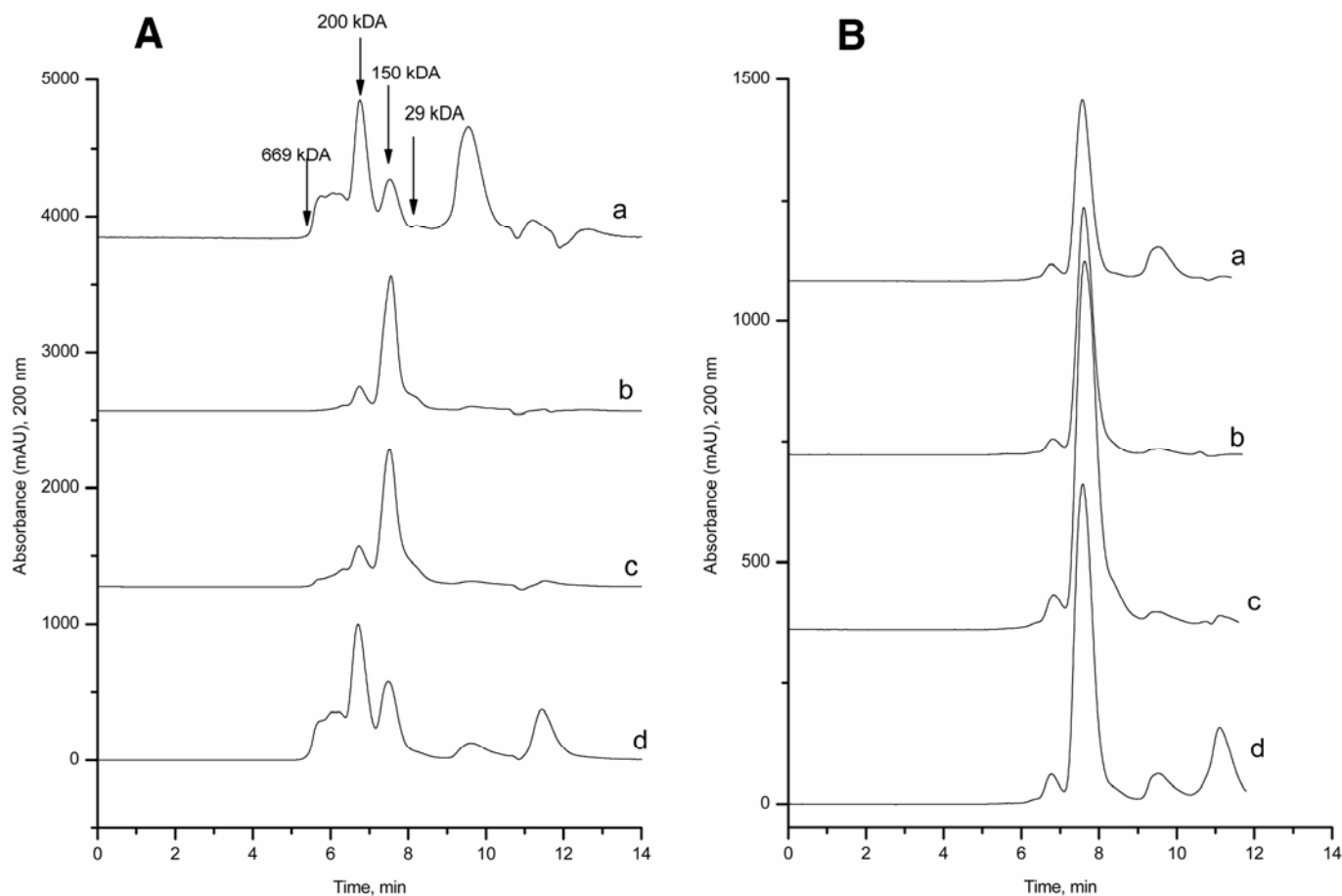


Fig. 3. SEC separations of selected protein samples initially extracted with a) 70% only and isolated by lowering ethanol to 50% with 1M NaCl added, b) 70% ethanol plus sodium metabisulfite and isolated by lowering ethanol to 50% with 1M NaCl, c) 70% ethanol plus glutathione and isolated by lowering ethanol to 30%, and d) 70% ethanol with sonication and isolated by lowering ethanol to 50% at pH 2.5. **A**, Samples redissolved without additional reducing agents. **B**, Samples redissolved with 2% β -ME added. Arrows indicated elution position of standard proteins used as molecular weight markers.

be optimized based on the initial extraction of the proteins, including the type of reducing agent used.

The data in Table I strongly indicate that compounds other than protein were being precipitated after the extraction. In some instances, high amounts of precipitate were found but the precipitate contained low protein. This was suggested not only by the data in Table I but also by the color of the precipitates. The original 70% ethanol extract of the grain was a light yellow. However, the color of the isolated precipitates varied from white to pale yellow to light red, depending on the precipitation conditions used (data not shown). This suggests compounds responsible for the various colors were affected by the precipitation. When using 1M NaCl or lowering precipitate proteins to pH 2.5, it is also possible that the proteins trapped salt molecules, which were then precipitated along with the proteins. Proteins precipitated using salts were more difficult to redissolve than proteins precipitated by manipulating ethanol concentrations alone. This may have been due to the salt present in the precipitated samples which, in turn, could affect the functionality of these protein fractions.

In this study, whole ground grain sorghum was used as a starting material, which included the bran (outer layers) of the sorghum grain. It is possible that materials in the bran, especially phenolic compounds, may interfere with the extraction of the sorghum proteins. da Silva and Taylor (2004) reported that kafirins extracted from sorghum bran had lower purity than kafirins extracted from sorghum endosperm and the protein extracted from bran was contaminated with polyphenols and lipids. Therefore, the purity of the proteins extracted in this study may have been reduced by using whole grain sorghum rather than decorticated sorghum.

Isolated proteins were resuspended and analyzed by RP-HPLC, FZCE, SEC, and SDS-PAGE to determine whether isolated proteins varied in surface hydrophobicity, charge density, or M_w . Such properties can influence protein functionality. Thus when the protein composition and properties of the various isolated proteins varied, it may be possible to adjust the precipitation procedures to prepare proteins for specific end-uses.

It was interesting that proteins from a given extraction condition showed little variation when precipitated under varying degrees of ethanol concentration (Fig. 2). Individual subclasses of kafirins have been defined based on variable solubility in different concentrations of aqueous 2-propanol (Shull et al 1991). For example, γ -kafirins were soluble in 10–80% tertiary butanol/ β -ME, α -kafirins soluble in 40–90% tertiary butanol/ β -ME, and β -kafirins soluble in 10–60% tertiary butanol/ β -ME. Differential solubility combined with precipitation from aqueous 2-propanol has also been used to purify kafirin subclasses (Watterson et al 1990; Shull et al 1991, 1992).

While minor differences were seen when a given extract was precipitated at different ethanol concentrations, major differences such as the complete absence of a particular subclass was not observed. This could have resulted from differences in kafirin solubility in aqueous ethanol versus aqueous 2-propanol. The use of additional conditions such as lowering the pH and adding NaCl could also modify kafirin solubility in aqueous ethanol and therefore alter how the kafirins behave in aqueous ethanol solutions. It should also be noted that solubility of cereal proteins can be much different than extraction of proteins from ground grain (Mifflin and Shewry 1979).

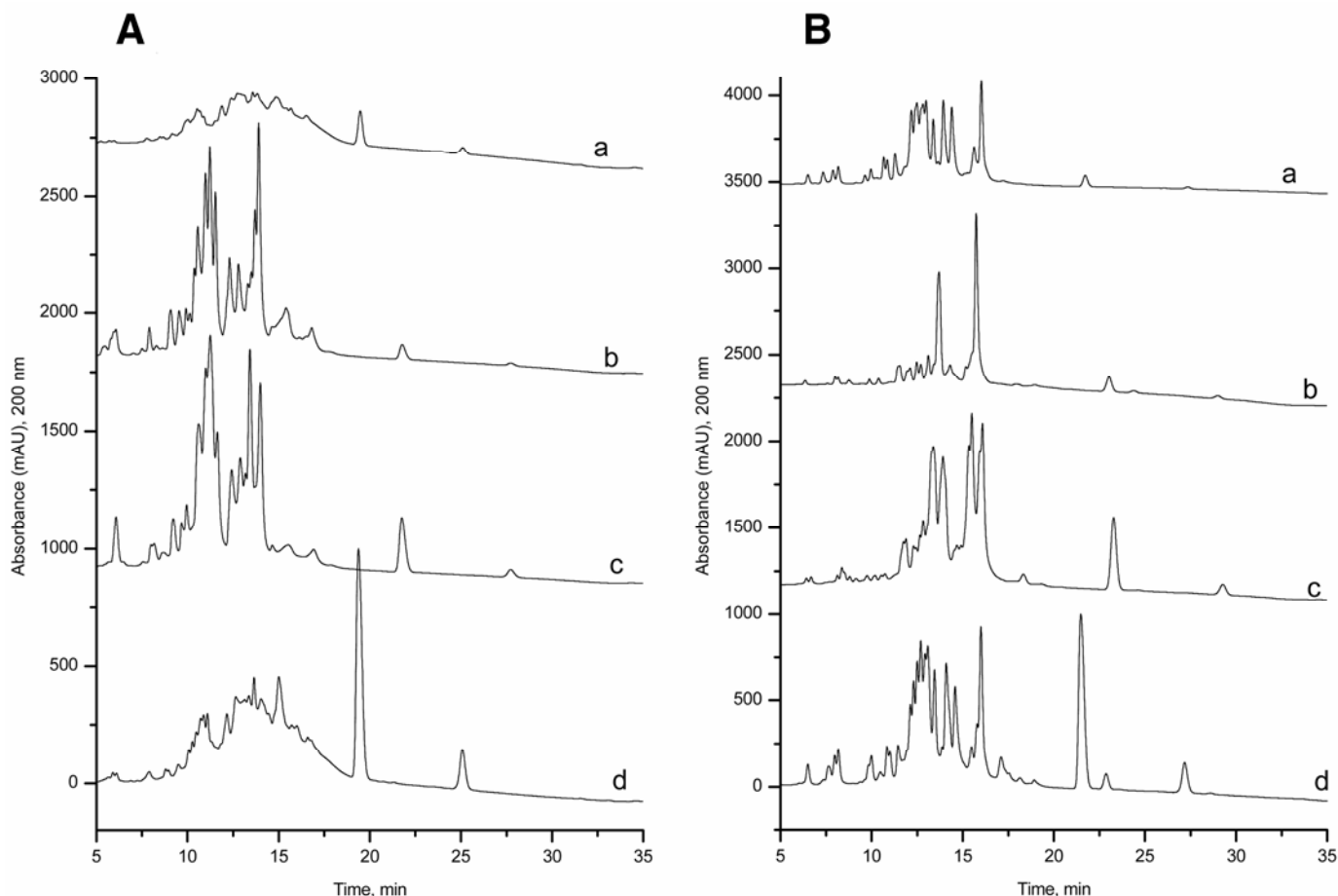


Fig. 4. RP-HPLC separations of selected protein samples initially extracted with a) 70% only, b) 70% ethanol plus sodium metabisulfite, c) 70% ethanol plus glutathione, and d) 70% ethanol plus sonication. **A,** Samples redissolved without additional reducing agents. **B,** Samples redissolved with 2% β -ME added.

Isolated protein fractions were analyzed both with and without additional reducing agents, where the only reduction of disulfide bonds would have occurred during the initial extractions, and with further reduction by the addition of β -ME to the isolated protein fractions. When analyzed without additional reduction via SEC, it was apparent that the various initial extraction conditions produced proteins with significant differences in M_w distributions. Both the 70% ethanol-only extract and the 70% ethanol/sonicated extract had wider molecular weight ranges than the other extracts. These samples also had the presence of proteins with M_w larger than individual kafirin proteins (≈ 20 – 30 kDa), indicating that polymeric proteins were solubilized. El Nour et al (1998) reported that sonication effectively solubilized polymeric proteins in sorghum, showing that such proteins do exist in sorghum. Nunes et al (2005) also studied polymeric proteins in sorghum through SDS-PAGE. The SEC data shown in Fig. 3A confirmed those results. However, when the proteins were reduced further with β -ME, all the samples showed similar M_w ranges, suggesting that the proteins had been reduced into individual subunits and that the larger proteins seen in the unreduced SEC chromatograms were polymeric proteins disulfide bonded together.

Differences were also found when the isolated protein fractions were analyzed by RP-HPLC (Fig. 4). The samples initially extracted with 70% ethanol only or in combination with sonication showed poorly resolved peaks spread over a broad range. However, as with the SEC analysis, once reduced with β -ME, the chromatograms between all the samples were similar. Differences were still present, suggesting that different kafirin subunits were extracted between the solvents used in the initial extraction.

Similar results were found during the FZCE analysis. It was interesting that additional peaks were seen in FZCE separations of the samples initially extracted with sodium metabisulfite that did not disappear once further reduced with β -ME. Currently, the identity of these peaks is not known.

The results of the SEC, RP-HPLC, and FZCE analysis showed that the initial extraction conditions had a significant effect on the biochemical properties (M_w , surface hydrophobicity, and charge density) of the subsequently isolated proteins. For example, the samples extracted without reducing agent showed the presence of polymeric proteins, which may have much different functionality than the proteins extracted with reducing agents. Thus the various protein fractions isolated in this project may have different functionalities when used in bio-industrial applications such as protein films or adhesives. They may also have unique functionality in gluten-free food products. Research is in progress to test these protein fractions in both bio-industrial and food applications.

CONCLUSIONS

Sorghum protein extraction methods originally used to prepare maize proteins for industrial uses were tested. Sorghum proteins prepared by these methods were evaluated with several types of analytical techniques. As expected, using a reducing agent in the initial protein extraction improved the amount and purity of the proteins isolated from the procedure. The conditions used to precipitate the proteins from solution influenced the quantity recovered and the purity of the fractions. The conditions yielding precipitates with the largest protein content varied according to the initial

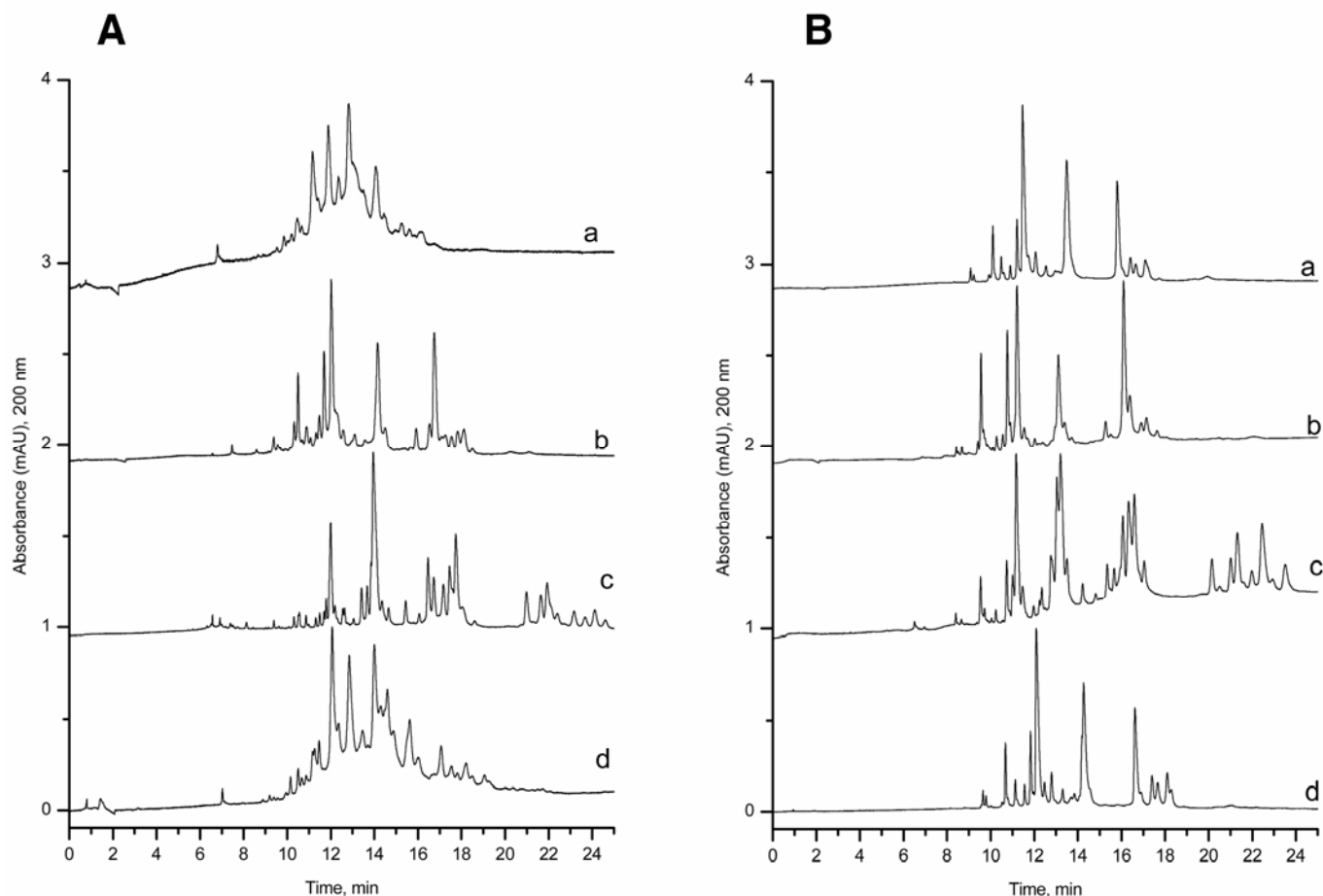


Fig. 5. FZCE separations of selected protein samples initially extracted with a) 70% only, b) 70% ethanol plus sodium metabisulfite, c) 70% ethanol plus glutathione, and d) 70% ethanol plus sonication. **A,** Samples redissolved without additional reducing agents. **B,** Samples redissolved with 2% β -ME added.

extraction conditions. This also influenced the biochemical properties of the isolated proteins, and thus provides an opportunity to produce proteins that may have unique functionalities for specific industrial applications.

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