Impact of different isolation procedures on the functionality of zein and kafirin ☆

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ABSTRACT

Commercial corn prolamin (zein) aggregates in water at elevated temperatures into an extensible, visco-elastic gluten-like substance. This specific functionality of zein can be used in the production of gluten-free bread from true dough systems and not from batters. The present study examined laboratory-scale isolation of such functional zein from dry milled corn. RP-HPLC indicated that successful isolation procedures resulted in relatively pure α-zeins, with a maximum ratio of $(\beta + \gamma)/\alpha$-zeins of about 10%. In the present study, such functional zeins were obtained by using 70% ethanol as the extractant, without added alkali or reducing agent in the main extraction step. In contrast, films could be cast from a wider range of zein isolates, also with higher ratios of $(\beta + \gamma)/\alpha$-zeins. Isolation of the analogous prolamin (kafirin) from dry milled sorghum required a more hydrophobic extractant such as 83% isopropanol to achieve partial functionality. Such kafirin was able to aggregate in warm water, preferably when a reducing agent was added; however, it quickly became firm and lost its extensibility. The present study suggests that hydro-phobic interactions rather than disulfide bonds are the key to gluten-like functionality of zein and kafirin.

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1. Introduction

The improvement of gluten-free bread remains a great challenge, as traditional gluten-free 'doughs' tend to be soft and batter-like, and require baking in pans (Schober et al., 2010). In contrast, a gluten-free system closely resembling wheat dough has been described by Lawton (1992). In this system, isolated maize prolamins (zein) and starch are mixed above zein's glass transition temperature (e.g. at 30–40 °C), resulting in viscoelastic, cohesive and extensible dough that contains gluten-like protein strands. Schober et al. (2008) successfully modified this zein-starch dough for breadmaking, by including the surface-active hydrocolloid hydroxypropyl methylcellulose (HPMC). In a subsequent study, this HPMC-containing zein-starch dough was improved by defatting the

surface of the zein particles which facilitated their aggregation, and promoted formation of gluten-like strands and stronger dough (Schober et al., 2010). The zein-starch dough in this latter study was for the first time suitable for the production of hearth-type gluten-free rolls, as it was strong enough to hold its shape during proofing and baking. The decisive prerequisite for the formation of zein-starch dough is the aggregation of zein particles in warm water into a gluten-like substance (Schober et al., 2008). In zein-starch dough, this aggregation can then lead to the zein-strands observed in the finished dough. All studies on zein-starch dough mentioned so far used commercial zein. Its production is relatively well-known, e.g., that on an industrial scale, it is isolated from corn gluten meal (a co-product of corn wet milling) using aqueous alcohols such as 86% isopropanol at elevated temperatures (e.g., 65 °C) (Carter and Reck, 1970; Shukla and Cheryan, 2001). During the wet milling process, corn is steeped in water containing sulfur dioxide (SO_2) which functions as a reducing agent while spontaneous lactic acid fermentation during steeping creates a low pH, helping to soften the grain (Du et al., 1996; Johnson and May, 2003; Wall and Paulis, 1978). As with all commercial processes, however, it appears a reasonable assumption that each company has further in-house knowledge not accessible to the public. Zein is produced by at least two large companies in the U.S. and Japan (Shukla and Cheryan, 2001), and numerous wet milling plants exist (Johnson and May, 2003).

Sorghum [*Sorghum bicolor* (L. Moench)] tends to be better adapted to hot, arid climates than corn, and its prolamins (kafirin) are similar to zein (DeRose et al., 1989; Shull et al., 1991). Like corn,

sorghum is considered safe for celiac patients and has long been used in gluten-free diets. A recent *in vitro* and *in vivo* challenge study confirmed that it is non-toxic for celiac patients (Ciacci et al., 2007). These facts make kafirin an interesting alternative to zein, and kafirin's similarity to zein suggests that it could possibly have similar functional properties, though kafirin's have not been as well studied as zein (Belton et al., 2006) However, in order to test for a gluten-like functionality of kafirin, a minimum of several grams are needed. Unlike zein, kafirin is not commercially available and would need to be isolated on a laboratory-scale. Such small-scale procedures have been used by many researchers to isolate zein from corn and kafirin from sorghum, however, they often differ considerably from the commercial zein isolation. For example, several studies used 70% (w/w) aqueous ethanol containing 0.35% sodium hydroxide (NaOH) and 0.5% sodium metabisulfite ($Na_2S_2O_5$) at 70 °C to extract kafirin directly from dry milled sorghum flour without previous steeping or wet milling (e.g. Da Silva and Taylor, 2004, 2005; Emmambux and Taylor, 2003; Gao et al., 2005). Most of these kafirin extraction procedures were not conducted to obtain kafirin for viscoelastic dough, but for the production of biodegradable films. Only one study (Oom et al., 2008) attempted the formation of kafirin-starch doughs, similar to zein-starch doughs. Raw material was a kafirin, isolated from sorghum with aqueous ethanol plus sodium metabisulfite; however, viscoelastic dough did not form and kafirin remained in the form of small particles in the surrounding starch and water. This finding raises the question whether any kafirin isolate would be unsuitable for the formation of viscoelastic kafirin-starch dough. Alternatively, it might have been that simply the specific isolation procedure used by Oom et al. (2008) resulted in kafirins that did not have the right properties for dough formation, and that a closer simulation of the commercial corn wet milling process would be required for kafirin isolation.

An interesting approach for a laboratory isolation procedure for zein was demonstrated by Parris and Dickey (2001). Dry milled corn flour was pretreated (steeped) in a variety of aqueous solutions, simulating effects of the wet milling process, before extraction with 70% ethanol. Steeping solutions included 0.5% sulfuric acid, 0.55% lactic acid plus 0.2% SO_2 and 0.5% sodium sulfite or bisulfite (Parris and Dickey, 2001). While the use of SO_2 and lactic acid is closest to commercial wet milling, an earlier study showed, that lactic acid can be replaced by a variety of other acids (including sulfuric acid) when used along with SO_2 (Du et al., 1996). Steeping of the already dry milled corn or sorghum may be a shortcut, providing similar effects as a complete laboratory-scale wet milling process in a much shorter time. Use of a combination of sulfuric acid and a sulfite salt is simpler than the combination lactic acid and SO_2. Parris and Dickey (2001) focused on zein films, and thus did not attempt zein aggregation in warm water for extensible, viscoelastic zein-starch doughs. This opens up the area for the present study:

First, crude kafirin was isolated from dry milled sorghum flour for preliminary characterization. The isolation procedure was then optimized for the isolation of zein from dry milled corn flour. A variety of laboratory procedures were used, including steeping similar to that of Parris and Dickey (2001). The main functionality test was the ability of these zein isolates to aggregate in warm water into a viscoelastic gluten-like substance. Procedures successful for zein isolation were then tested for the isolation of kafirin with improved functionality to find an optimized kafirin isolation procedure.

2. Experimental

2.1. Materials

Corn meal (yellow, whole grain) was obtained from Hodgson Mill, Effingham, IL. It was reground into fine powder ('corn flour')

with a Cyclone sample mill (Udy, Fort Collins, CO) equipped with a 1 mm-screen. Sorghum flour was from Twin Valley Mills, Ruskin, NE. Protein content (N × 6.25, wb, AACC Method 46-30; AACC, 2000) was 7.3% and 6.4% for the corn and sorghum flour, respectively.

Commercial zein was obtained from Sigma–Aldrich (St. Louis, MO). This specific lot (057K0156) has been extensively characterized in a previous study (Schober et al., 2010). In the present study, it was defatted (3 times with hexane) as described for the lab-scale zein isolation process. It was included as a control in RP-HPLC, SE-HPLC and for the preparation of cast films.

2.2. Zein isolation

Twelve different treatments for zein isolation were compared (Table 1). Duplicate batches of corn flour (300 g each) were each suspended in 1200 ml extractant [70% v/v ethanol/water; specific amounts of sodium metabisulfite ($Na_2S_2O_5$) and/or sodium hydroxide (NaOH) were added for some treatments as detailed in Table 1; unless described differently, $Na_2S_2O_5$ and NaOH were mixed with the extractant before its addition to the flour]. Extraction was conducted for 1 h, with stirring every 15 min, at temperatures of 50 or 70 °C (Table 1). A 1 h rest period at room temperature was allowed for sedimentation of the bulk of flour particles. The supernatant was then centrifuged (10 min at $3200 \times g$) to remove remaining particles. Then, solubilized proteins were precipitated by a combination of dilution (from 70% to 50% ethanol/water), cooling (−20 °C, over night), and, for some treatments, pH adjustment (Table 1). The precipitated protein was collected by centrifugation (10 min at $3200 \times g$), frozen at −80 °C and freeze-dried. The dry protein was then ground into fine powder, defatted (2.5 ml hexane/g powder, 3 times for 1 h at room temperature with continuous stirring followed by decanting) and residual hexane evaporated under vacuum.

If presoaking was done, then the following steps preceded the above extraction (modified from Parris and Dickey, 2001): each 300 g of corn meal was mixed with 1500 ml of an aqueous solution containing 0.3% sodium metabisulfite and 0.5% sulfuric acid. The suspension was incubated for 6 h at 50 °C with occasional stirring, followed by decanting of this steeping solution and two washing steps (1500 and 1200 ml distilled water, respectively). After the second wash, the soaked flour was drained for several hours and then weighed. Increase in weight (residual water) was accounted for in the subsequent extraction by increasing alcohol concentration, so that the overall concentration of extractant plus residual water remained 70% ethanol.

2.3. Preliminary kafirin isolation and rheology

For preliminary characterization, crude kafirin was directly isolated from sorghum flour, following the procedure described for zein isolation, using treatment 3 (Table 1; 70% ethanol, 0.35% NaOH, 0.5% $Na_2S_2O_5$, extraction at 70 °C, precipitation at pH ≈ 2.5). For initial characterization, the thermal behavior of this hydrated crude kafirin was studied by fundamental rheology. For this purpose, crude kafirin (2 g) was prewarmed to 60 °C and then mixed with 4 ml water (60 °C). This dispersion was centrifuged (2 min at $3200 \times g$) and a 2 g portion of the pellet inserted in a rheometer. This was the same instrument described previously (Schober et al., 2010): a Reologica ViscoAnalyser 50 (ATS RheoSystems, Bordentown, NJ) equipped with a pressurized sealed cell (to prevent sample drying), in combination with a serrated plate measuring system (20 mm diameter). Dynamic oscillatory temperature sweeps were conducted with the following settings: autotension (normal force maintained at 1×10^{-2} N, gap variable in the range

Table 1
Treatments for zein isolation, and their characterization.

Treatment	Presoaking[a]	Extractant[b]	Extraction temperature (°C)	Precipitation pH[c]		Functionality (aggregation in 40 °C water?)[d]	Protein (N × 6.25), %	Lipid, %
1	–	0.35% NaOH[b], 0.5% Na$_2$S$_2$O$_5$[b]	50	≈2.5	–	Paste-like dispersion	82.7	13.9
2	–	0.5% Na$_2$S$_2$O$_5$	50	≈2.5	0	Aggregation into a brittle solid, increasingly cohesive over 15 min 'tendency towards strands'	75.1	17.8
3	–	0.35% NaOH, 0.5% Na$_2$S$_2$O$_5$	70	≈2.5	–	Paste-like dispersion	85.2	11.2
4	–	0.5% Na$_2$S$_2$O$_5$	70	≈2.5	0-	Resembles treatment 2, but less increase in cohesiveness over the 15 min	75.4	18.0
5	–	0.35% NaOH, 0.5% Na$_2$S$_2$O$_5$	50	≈5	–	Paste-like dispersion	86.9	10.8
6	–	0.1% NaOH[b], 0.5% Na$_2$S$_2$O$_5$	50	≈5 (not adj.)	–	Paste-like dispersion, coarse particles, 'sandy'	82.3	12.4
7	–	0.5% Na$_2$S$_2$O$_5$	50	≈5	–	Paste-like dispersion, coarse particles, 'sandy'	81.6	14.2
8	–	– (70% ethanol only)	50	≈5	+	Immediately cohesive > aggregation, upon extension: firm, stiff, but forms strands	75.4	17.1
9	Yes	– (70% ethanol only)	50	≈5	0+	Like treatment 8, but some remaining coarse particles, elastic	89.3	4.8
10	Yes	NaOH to pH ≈8 (≈0.2%)[e]	50	≈5	0-	Paste-like & gritty, but slightly cohesive	85.0	11.6
11	–	0.1% NaOH (added last), 0.5% Na$_2$S$_2$O$_5$[f]	50	≈5 (not adj.)	–	Paste-like dispersion, coarse particles, 'sandy'	79.2	14.0
12	Yes	– (70% ethanol only)	50	≈4 (not adj.)	++	Immediately cohesive > aggregation, upon extension: films & strands, changes from stiff to smooth during 15 min at 40 °C	66.8	24.8

[a] Presoaking: in water containing 0.3% Na$_2$S$_2$O$_5$ and 0.5% H$_2$SO$_4$; 6 h at 50 °C, followed by washing with water.
[b] Basis for all extractants: 70% v/v ethanol. For 100 ml extractant: 70 ml ethanol; as needed: 0.5 g Na$_2$S$_2$O$_5$, 0.7 ml and 0.2 ml 50% NaOH for 0.35% and 0.1% NaOH, respectively; to 100 ml with water.
[c] pH in the presence of 50% ethanol estimated with pH paper; adjustment with HCl or NaOH; if 'not adj.', then pH estimated, but not adjusted.
[d] Functionality: –, -, 0-, 0, 0+, +, ++ describes increasingly desirable functionality (desirable: aggregation, smoothness, formation of gluten-like strands).
[e] Tr. 10: First ethanol added to presoaked flour, then pH adjusted to ≈8 with 50% NaOH (resulting composition of extractant: 70% v/v ethanol, ≈0.2% NaOH).
[f] Tr. 11: Ethanol, Na$_2$S$_2$O$_5$ and water mixed with corn flour; NaOH added last; then incubation as usual.

2.1–2.3 mm); 1 Hz, 1 data point per minute, target strain 1×10^{-3}. (The target strain of 1×10^{-3} was in the linear viscoelastic region based on stress sweeps.) A temperature profile was applied, which was expected to cover glass transition (10 min at 60 °C, followed by a linear gradient of 1 °C/min down to 25 °C. A holding period at 25 °C for 10 min followed, and then a gradient of 1 °C/min back up to 60 °C; finally, the temperature was held constant for 10 min at 60 °C).

2.4. Optimized kafirin isolation

Final, optimized kafirin isolation was based on the results obtained for zein isolation. Kafirins were extracted from sorghum flour in two different ways. One treatment followed exactly treatment 12 (Table 1); the second treatment for kafirin isolation was a modification of treatment 12: 83% v/v isopropanol (IPA) rather than 70% ethanol was used as the extractant. Additionally, this isopropanol extract was diluted to 40%, rather than 50%, to aid precipitation at −20 °C. The resulting kafirins had very low protein contents (46–52%, N × 6.25) and felt clearly fatty; therefore, a portion of each was further defatted with chloroform (10 ml CHCl$_3$/g powder, 3 times for 1 h at room temperature with continuous shaking followed by decanting) and residual chloroform evaporated under vacuum in the freeze-drier. This latter defatting step increased the protein content to 79% and 84% for ethanol and isopropanol-extracted kafirin isolate, respectively. These kafirin isolates are abbreviated Kaf$_{EtOH}$ and Kaf$_{IPA}$ in this article.

2.5. Protein and lipid content of isolates

Protein content (N × 6.25) of zein and kafirin isolates was determined by combustion (AACC Method 46-30; AACC, 2000).

Lipid content of the zein isolates was determined in duplicate from 1 g sample, respectively, by accelerated solvent extraction (ASE) at 100 °C and 69 bar with chloroform as described previously (Schober et al., 2010). The reported % values for protein and lipid content correspond to a dry basis.

2.6. Functionality tests

Zein in an excess of water (e.g., 2.2 ml water/2 g zein) aggregates at temperatures above its glass transition temperature of ≈29 °C (Schober et al., 2008), while heat-induced cross-linking does not start below ≈59 °C (Schober et al., 2010). Preliminary experiments indeed confirmed that commercial zein aggregates in an excess of water at 40 and at 55 °C. In agreement with previous studies (Schober et al., 2008, 2010), zein aggregation was tested at 40 °C. Preliminary rheological tests, however, suggested a higher glass transition temperature of kafirin than zein (above 40 °C, see below and Fig. 1). Thus, aggregation tests with kafirin were conducted at 55 °C. In all cases, isolates (zein or kafirin) and water were pre-warmed to the test temperature. Water (2 ml/g isolate) was added, mixed with the isolate, the resulting dispersion or aggregate observed during 15 min at the test temperature and described qualitatively. For kafirin only, additionally to water, the effect of water containing 5% (v/v) 2-mercaptoethanol was tested.

2.7. Resolubilization of aged, aggregated kafirin (aged Kaf$_{IPA}$)

An additional test was conducted with the isopropanol-extracted, chloroform-defatted kafirin (see Optimized kafirin isolation, Kaf$_{IPA}$). As before, it was mixed at 55 °C with water containing 5% 2-mercaptoethanol (2 ml/g Kaf$_{IPA}$). The aggregated kafirin was removed from the excess of water plus mercaptoethanol, and attempted to load between the plates of the rheometer.
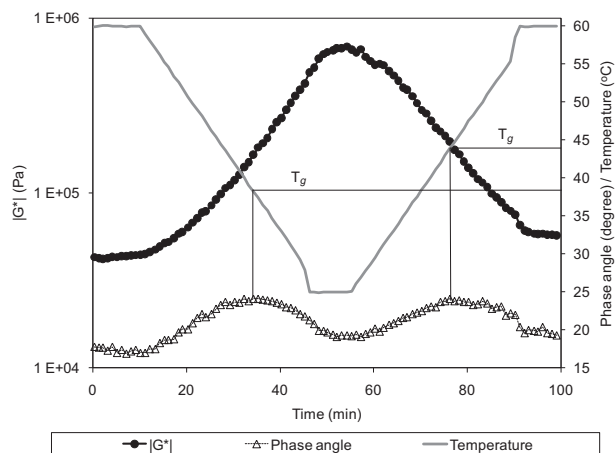
Fig. 1. Dynamic oscillatory temperature sweep with hydrated crude kafirin particles. Glass transitions (T_g) at maximum phase angle are marked.

However, within minutes, before the loading could be completed, this aggregated kafirin became so firm and elastic that it could no longer be pressed between the plates, but remained a thick disk not allowing further reduction of the gap. In attempts to reduce the gap sufficiently to flatten this disk and thus allow complete filling, the aggregated Kaf$_{IPA}$ sample was kept in the rheometer, at 55 °C, over night, surrounded by water containing 5% 2-mercaptoethanol. Subsequently, it was submerged into excess water plus 5% 2-mercaptoethanol at 55 °C to possibly allow swelling. Then, attempts to load the sample into the rheometer were repeated, however without success.

In order to study the possible reason for this extremely strong aggregation, four days after the initial aggregation in water plus mercaptoethanol, resolubilization of this aged, aggregated kafirin (aged Kaf$_{IPA}$) at room temperature in a more hydrophobic solvent was attempted. For this purpose, the aged Kaf$_{IPA}$ was ground with a mortar and pestle, and 60% *tert*-butanol plus 0.5% sodium acetate added (50 ml of the aqueous butanol for 0.18 g aged Kaf$_{IPA}$, no reducing agent added). After 1 h, 1 ml 2-mercaptoethanol was added (resulting in a concentration of 2% v/v) and the gradual dissolution of the kafirin observed during the following 10 days. Then, another ml of 2-mercaptoethanol was added and further dissolution observed during 6 more days.

2.8. Preparation of cast films

Cast films were prepared in a similar way as described by Parris and Dickey (2001). Zein isolate (1.00 g) was dispersed in 30 ml of aqueous ethanol (90% v/v) and heated for 10 min in a water bath set to 79 °C to reach a final sample temperature of 70 °C. The supernatant containing solubilized zein was separated from insoluble particles by centrifugation (3 min at 3200 × g), poured in a tared plastic Petri dish and dried at 50 °C to constant weight (38 h). The final weight of the dried films was recorded, and film yield reported as [g cast dry zein film from 1.0 g zein isolate]. A portion of each film was removed from the Petri dish and ground into powder for RP-HPLC analysis.

2.9. RP-HPLC

Zein isolates and zein films were solubilized in a buffered guanidine system under reducing conditions, followed by alkylation with 4-vinylpyridine (modified from Crankshaw and Grant, 1996). Briefly, zein isolates or ground cast zein films (5 mg) were solubilized in 1 ml 1 M TRIS-HCl buffer pH 8.5 containing 4 mM

EDTA (1 part by volume), and 8 M guanidine HCl (3 parts by volume) and 2% (v/v) 2-mercaptoethanol with continuous shaking for 30 min, followed by centrifugation (16,000 × g, 4 min). An aliquot was then incubated with 6.7% (v/v) 4-vinylpyridine for 15 min with shaking, followed by centrifugation (16,000 × g, 4 min). This zein solution (5 μl) was injected into an Agilent 1100 HPLC system, equipped with an Agilent Poroshell 300SB-C18 column (2.1 × 75 mm). A binary gradient was run at a constant flow rate of 0.7 ml/min, with Solvent A = water + 1 g/l trifluoroacetic acid (TFA), Solvent B = acetonitrile + 0.7 g/l TFA. Gradient time-table: at 0 min, 80/20 (%A/%B); at 5 min, 60/40; at 15 min, 40/60; at 17 min, 80/20; at 18 min, 80/20. The column temperature was maintained at 55 °C, and detection was done at 214 nm (Bean et al., 2010). This method was also tested with 5 mg Kaf$_{EtOH}$ and Kaf$_{IPA}$.

2.10. Preparation of standard zein fractions

Standards were prepared to identify the elution times of β- and γ- versus α-zeins, making use of the solubilities described by Esen (1987). First, the corn flour was pre-extracted twice with 50 mM TRIS-HCl buffer, pH 7.8 containing 0.1 M KCl and 5 mM EDTA, followed by two extractions with distilled water (all pre-extractions for 5 min with stirring and 4 ml solvent/g flour; these pre-extractions remove albumins, globulins and then residual salts; Bean and Lookhart, 1998). The pre-extracted flour was then freeze-dried. Subsequently, α-zein was prepared by extracting this freeze-dried flour with 90% (v/v) isopropanol (30 min with stirring; 4 ml/g flour; two subsequent extractions). γ zeins were isolated from the pre-extracted, freeze-dried flour in the same way; however, 20% (v/v) isopropanol containing 2% 2-mercaptoethanol was used for extraction, while the combined (β + γ) zeins were extracted with 35% (v/v) isopropanol containing 2% 2-mercaptoethanol. All extracts were then freeze-dried. Both, the γ- and the (β + γ)-zeins were further purified by two subsequent extractions with 92% (v/v) isopropanol to remove residual α-zeins; 92% isopropanol was applied in high excess (>10 ml/g) with shaking for 30 min, followed by freeze-drying. Finally, all zein fractions were defatted three times for 30 min with an excess (>10 ml/g) of hexane.

2.11. Size-exclusion (SE)-HPLC

SE-HPLC was conducted in order to compare size distribution of the non-reduced zein isolates. For this purpose, zein isolate (5 mg) was extracted for 30 min with continuous shaking, using 1 ml of 12.5 mM Na-borate buffer, pH 10, containing 2% sodium dodecyl sulfate (SDS). Then, the sample was centrifuged (16,000 × g, 4 min) and verified for presence of a precipitate. Subsequently, 15 μl of the supernatant was injected in the SE-HPLC system and analyzed as described previously (Schober et al., 2007).

2.12. Protein hydropathicity predictions

Deduced kafirin and zein amino acid sequences were obtained from UniProt (The UniProt Consortium, 2009). The specific sequences used in this publication were as follows: α-kafirin, Uni-Prot ID Q8LJY6; β-kafirin, UniProt ID Q6KB71; and γ-kafirin, UniProt ID C5XDL2. Zein sequences used were: α-zeins, UniProt Q00919; β-zein, UniProt ID Q946W0; and γ-zein, UniProt Q548E9. The locations of hydrophobic regions in the amino acid sequences obtained above were predicted using the Kyte-Doolittle hydrophobicity scale (Kyte and Doolittle, 1982) from the Protein Hydroplotter (http://www.proteinlounge.com/). The overall hydrophobicity (grand average of hydropathy - GRAVY) was also calculated using the Kyte-Doolittle method (Kyte and Doolittle, 1982) via ProtParam

(http://expasy.org/tools/protparam.html). Signal peptides were not included in any calculations.

3. Results and discussion

3.1. Crude kafirin

Crude kafirin was isolated from dry milled sorghum flour similar to methods previously described [Table 1, treatment 3: no presoaking, 70% ethanol, 0.35% NaOH, 0.5% $Na_2S_2O_5$, 70 °C; compare Da Silva and Taylor (2004, 2005); Emmambux and Taylor (2003); Gao et al. (2005)]. This crude, powdery kafirin clearly did not aggregate in water into a gluten-like substance, even though a wide range of temperatures was tested (40, 50, 60, 70, 85 °C). Individual kafirin particles (originating from grinding of the freeze-dried protein precipitate during isolation) appeared to be simply dispersed in water, forming a paste. However, it was observed that during heating in water, these individual particles became softer and more rubbery in comparison to the dry powder. When the hydrated particles were collected by centrifugation and subjected to dynamic oscillatory temperature sweeps between 25 and 60 °C, indeed glass transition at around 40 °C was visible (Fig. 1). Differences between values for the glass transition temperature (T_g) measured during downward and upward temperature gradient are most likely because of delayed heat transfer, resulting in the sample temperature lagging behind the temperature registered by the instrument. The true value of T_g is therefore likely between these two measured values (Schober et al., 2008), at ≈41 °C. In order to be clearly above T_g, subsequent aggregation tests with kafirin were conducted at 55 °C.

3.2. Zein isolation

If treatment 3 (Table 1) was applied to corn flour, the isolated crude zein also produced a paste-like dispersion in warm (40 °C) water instead of functional, gluten-like aggregated zein (Table 1). Thus this specific isolation procedure is not suitable for producing functional kafirin or zein (functional here being defined as able to aggregate in warm water into a gluten-like substance). For further attempts to isolate zein, therefore, the original extraction procedure was varied, leading to the 12 treatments in Table 1. Modifications included: lowering the extraction temperature from 70 °C to 50 °C (all treatments except No. 3 and 4); applying a less acidic pH for precipitation [pH 4–5 rather than 2.5, in line with Da Silva and Taylor (2004, 2005); Emmambux and Taylor (2003); Gao et al. (2005)]; testing extraction with $Na_2S_2O_5$ but without NaOH (Gao et al., 2005), or with lower levels of NaOH. It was observed that the 0.5% $Na_2S_2O_5$ and 0.35% NaOH in combination formed a white precipitate in 70% ethanol. Stirring of this extractant was required to maintain a homogenous suspension. Both $Na_2S_2O_5$ and NaOH individually were soluble in 70% ethanol. The presence of insoluble sulfites or NaOH in 70% ethanol, when adding both $Na_2S_2O_5$ and NaOH simultaneously might create locally very high concentrations and overall inhomogeneous conditions. Thus, a different sequence of addition was tested, in which NaOH was added to the flour suspension last, after the other components of the extractant (Treatments No. 10 and 11). Similarly, the sequence of solvent addition has been found important by Concon (1973), and these authors suggested to first mix 70% ethanol with corn flour, then add NaOH. Simulating wet milling, presoaking with a mixture of 0.3% $Na_2S_2O_5$ and 0.5% H_2SO_4 was also tested. Most important, use of 70% aqueous ethanol as the sole extractant, without any added NaOH or $Na_2S_2O_5$ was tested (Treatments 8, 9 and 12), with or without presoaking. These three latter treatments were the only ones that produced functional zein, able to aggregate in warm

water into a gluten-like substance and form strands (Table 1). Extraction temperature, precipitation pH, or sequence of addition of NaOH had no effect on functionality. The presence of either $Na_2S_2O_5$ or NaOH in the extractant created non-functional zein (Table 1), whereas, reducing conditions ($Na_2S_2O_5$) in the presoaking step had slightly beneficial effects (Table 1, Tr. 12). For a better understanding of these observations, RP-HPLC was used to separate and quantify zein classes. Using purified standards, Fig. 2 shows that α-zeins were well-separated from β and γ-zeins. This figure also suggests that the functional treatment 12 was composed mainly of α-zeins, while the non-functional treatment 6 contained a high percentage of β and γ-zeins (Fig. 2, Table 1). In order to investigate this observation for all treatments, the ratio (β + γ)/α-zeins was calculated for all isolates in Table 1. The ranges marked in Fig. 2 were used for integration of the RP-HPLC runs. All functional zeins (No. 8, 9, and 12) were characterized by very low contents of β and γ zeins (Fig. 3). In contrast, the three treatments rated worst in Table 1 (No. 6, 7, and 11) were characterized by the highest contents of β + γ zeins. All three were rated '–' because they yielded sandy pastes in warm water composed of coarse particles. Additionally, Fig. 3 shows that the protein yield and the ratio (β + γ)/α-zeins were highly correlated. Therefore, in this study a higher yield was only achieved by coextracting more undesirable β and γ-zeins together with the desired α-zeins.

β and γ-zeins differ from α-zeins in two important characteristics: they are more hydrophilic, indicated by their solubility in more dilute aqueous isopropanol (Esen, 1987) or more dilute acetonitrile (Bean et al., 2000). This is also in agreement with their earlier elution in RP-HPLC (Fig. 2). Furthermore, β and γ-zeins contain more cysteine than α-zeins, require a reducing agent for their extraction, and tend to form disulfide-linked oligomers or polymers (Esen, 1987; Parris and Dickey, 2001). Commercial zeins are mainly α-zeins, with only small amounts of other zeins, as qualitatively determined by SDS-PAGE (Zhu et al., 2007). The present study suggests that a ratio of (β + γ)/α-zeins of ≈ 10% is the
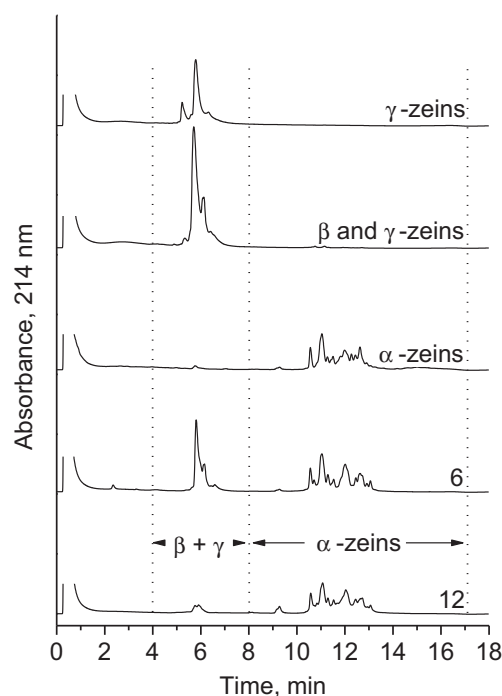

Fig. 2. RP-HPLC with purified zein types (γ, β and γ, and α-zeins) and zein isolates (treatment 6 and 12, Table 1). Vertical, dotted lines indicate ranges used for integration of peak areas considered (β + γ) and α-zeins, respectively. Void peaks (<2 min) truncated.

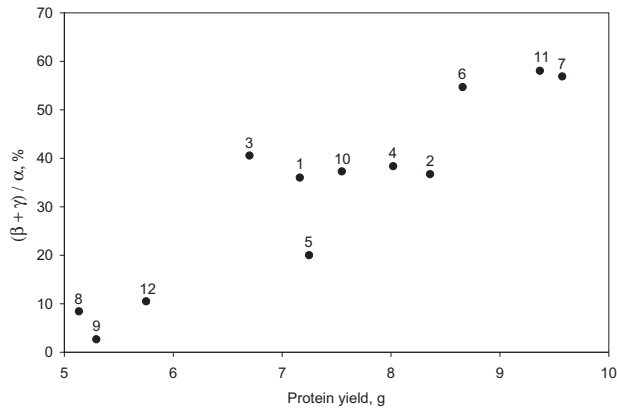**Fig. 3.** Relationship between protein yield [g protein (N × 6.25) from 600 g corn flour] and the ratio of (β + γ) to α-zeins (based on RP-HPLC areas). Twelve different isolation procedures are compared, and the data points are labeled with treatment number (Table 1). Correlation coefficient between the two plotted variables is 0.93 ($P < 0.001$).

Fig. 4. Relationship between film yield [g cast dry zein film from 1.0 g zein isolate] and the ratio of (β + γ) to α-zeins in the isolates (based on RP-HPLC areas). Twelve different isolation procedures are compared, and the data points are labeled with treatment number (Table 1). Correlation coefficient between the two plotted variables is −0.80 ($P < 0.01$).

maximum for functionality (i.e., aggregation in warm water into a gluten-like substance; Fig. 3; Table 1).

3.3. Zein film formation

Zein isolates from all 12 treatments (Table 1) and commercial zein could be used to make cast films. These films were cohesive, had a smooth surface and either formed a coherent coating on the Petri dish that could only be removed with difficulty, or came off as a coherent film. During film preparation, zein isolates were solubilized in hot 90% ethanol and insoluble fractions removed. This resulted in an overall decrease of β + γ zeins in the films relative to the original zein isolates. For treatment 1 to 12, the ratio (β + γ)/α-zeins changed from isolate to film as follows: Treatment 1: 36% for isolate, 16% for film; Tr. 2: 37% (isolate), 28% (film); Tr. 3: 41%, 15%; Tr. 4: 38%, 18%; Tr. 5: 20%, 11%; Tr. 6: 55%, 24%; Tr. 7: 57%, 26%; Tr. 8: 8%, 7%; Tr. 9: 3%, 2%; Tr. 10: 37%, 32%; Tr. 11: 58%, 31%; Tr. 12: 11%, 7%. Commercial zein and its film both had the identical, low (β + γ)/α-ratio of 6% (all values based on RP-HPLC areas).

As β + γ zeins were preferably removed, i.e. α-zeins enriched in the films, it is not surprising that a negative correlation was found between the ratio (β + γ)/α-zeins in the isolates, and the film yield (g dry film from 1.0 g zein isolate; Fig. 4). Also as expected, treatments 8, 9, and 12 achieved the highest film yields. However, even though the ratio (β + γ)/α was lower in the films than in the respective isolates, it was still distinctly higher than 10% in the majority of the films. This indicates that film formation is less sensitive to the presence of β and γ zeins than aggregation in warm water. It appears reasonable to assume that in the films, excessive α-zeins (easily soluble in 90% ethanol) simply embed β and γ zeins during casting.

3.4. Size distribution of non-reduced zein isolates

Size distribution was determined with the fraction of each zein isolate that was soluble in non-reducing, alkaline, SDS containing buffer (SDS-soluble fraction). However, for several of the isolates, a distinct insoluble pellet (precipitate) remained (treatments 6, 7, and 11), while for the remaining treatments and commercial zein, a precipitate was absent or too small to be clearly visible. The non-reducing, alkaline, SDS containing buffer can be expected to cleave all types of bonds between side chains from amino acid residues in zein (particularly hydrophobic bonds because of the amphiphilic nature of SDS), except for disulfide bonds. Zein isolates from treatments 6, 7, and 11 were highest in β + γ zeins (Fig. 3), and have
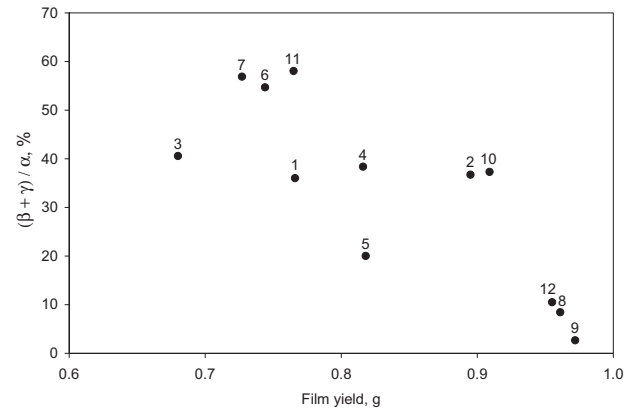
thus the highest potential to form disulfide bonds. It appears therefore a reasonable assumption that the SDS-insoluble precipitates observed for these treatments were disulfide-linked proteins. Disulfide bonds in these proteins could cause both, relatively high molecular weight, and abundant cross-linking. Cross-linking may result in limited access for water and SDS and thus limited swelling and solvation. Such large cross-linked proteins may explain the particularly 'sandy' dispersions formed by these treatments in warm water (Table 1). The size distribution of the SDS-soluble fraction (Fig. 5) appears to be a poor indicator of zein's functionality. Within the treatments shown in Fig. 5, treatments 8, 12 and the commercial zein were able to aggregate in warm water (Table 1). However, all three differed in their size distribution; from narrow (commercial zein) via slightly wider (Tr. 12) to very broad (Tr. 8); with Tr. 8 including substantial amounts of larger molecules eluting at shorter times (<7 min). Non-functional isolates ranged from very narrow (Tr. 10) to very broad (Tr. 6, including SDS-insoluble precipitate). These overall observations suggest some importance of disulfide bonds: SDS-insoluble, disulfide-linked proteins appear to be undesirable (treatments 6, 7, and 11), while a wider size-range of soluble disulfide-linked proteins (Tr. 8) seems to not interfere with the functionality.

3.5. A model for zein aggregation in water

Protein aggregation can be impacted by covalent (disulfide) and non-covalent bonds. Protein structure is also an important factor in the aggregation of proteins. Substantial research has been conducted to determine the structures of zeins in solution and in films, and how these proteins might aggregate. This is especially true of the α-zeins for which detailed structures have been reported. The α-zeins have been found to form a series of anti-parallel α-helices in the shape of a "ribbon" (Lai et al., 1999; Matsushumi et al., 1997). Individual α-zeins are thought to aggregate together into oligomeric structures representing "prisms" where "ribbons" from individual α-zeins aggregate together through the hydrophobic surfaces of the α-helix "ribbons" with each oligomeric structure made up of four or more individual α-zeins (Lai et al., 1999; Matsushumi et al., 1997). These structures are stable and are not disrupted during processing of zein (Wang et al., 2005).

During film formation, zein has been reported to form large globular structures that vary in size. Size variability of the globules was speculated to be caused by varying degrees of aggregation of the proteins (Guo et al., 2005). Using atomic force microscopy, Guo
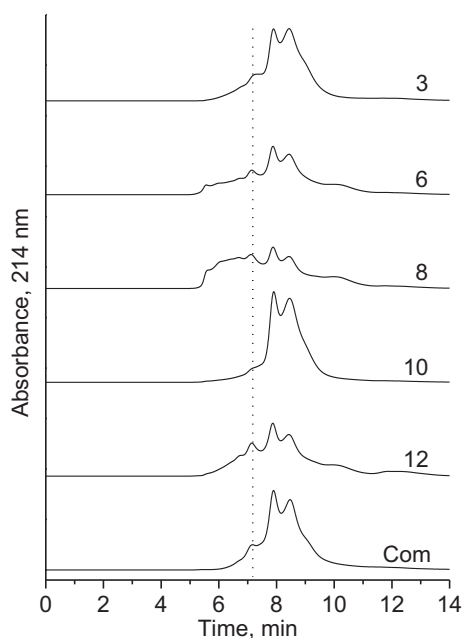
Fig. 5. SE-HPLC with zein isolates solubilized in 12.5 mM Na-borate buffer, pH 10, containing 2% SDS. Numbers indicate treatment (Table 1), while 'Com' is a commercial zein for control purposes. The vertical, dotted line indicates the elution time of the first distinct peak in all treatments.

et al. (2005) found that large zein aggregates (globules) formed a mesh-like structure when evaporated onto a mica surface. Kim and Xu (2008) expanded this research by following the aggregation of zein in solution using dynamic light scattering, thus avoiding any artifacts created by the zein being evaporated onto the mica surface in the work of Guo et al. (2005). A detailed description of how zein might aggregate when evaporated into films was provided by Kim and Xu (2008). These authors characterized zein as amphiphilic and assumed that its aggregates orient their hydrophilic moieties towards the solvent medium if the solvent is more polar than 90% ethanol or towards the center of the zein aggregate if the solvent is less polar than 90% ethanol.

This may be relevant for the aggregation of zein in dough systems. For dough formation, zein is first mixed with warm water where upon the proteins would rearrange to expose hydrophilic surfaces and bury hydrophobic surfaces, as pure water is much more polar than the threshold of 90% ethanol (Kim and Xu, 2008). As discussed previously, it is thought that for α-zeins, aggregation of single proteins into oligomeric complexes occurs through the hydrophobic surfaces of the α-helical 'ribbons' (Lai et al., 1999; Matsushumi et al., 1997). Physical interaction of zein particles during mixing would tend to drive the formation of larger aggregates by bringing zein particles into physical contact with each other, thus minimizing the ratio of surface area to volume. This would result in increasingly large aggregates with the most hydrophobic domains in the inside and the less hydrophobic moieties exposed to the surrounding water at the surface of the aggregates.

The functionality of the zein isolates prepared in this work suggests that disulfide bonds are not responsible for zein aggregation in warm water into a macropolymer able to form gluten-like strands. Disulfide bonds could have a negative effect, as they might prevent aggregation of the α-zein helical structures. The presence of β-zeins in the protein bodies of maize has been found to alter the composition of the secondary structure of zein complexes (Forato et al., 2003). While the work of Forato et al. (2003) was carried out on solid materials, the secondary structures reported matched

those reported for zeins in solution (in fact this data was used to confirm that structural measurements made in solution match those found in the grain). Thus it is plausible that disulfide cross-links interfere with the aggregation of zeins during dough mixing in such a way as to reduce the functionality of the proteins.

Therefore, formation of zein-starch dough systems may be more reliant on non-covalent interactions and not covalent interactions such as disulfide cross-linking. This is particularly interesting due to zein's pronounced hydrophobicity relative to many other cereal prolamins (Belitz et al., 1986; Chiue et al., 1997), and because of the effects of lipids on the aggregation of zein in water (Schober et al., 2010). Removal of surface lipids resulted in improved dough properties of zein-starch mixtures, potentially by preventing aggregation of zein proteins. Surface lipids may have been bound to the hydrophobic planes of the α-zein 'ribbons' reducing protein–protein aggregation. Such protein–lipid interactions have been reported in zein-lipid films (Lai et al., 1999; Wang et al., 2005).

3.6. Optimized kafirin isolation

Kafirins were isolated from sorghum flour following treatment 12 (Table 1, 70% ethanol for isolation), and a modified treatment 12 (83% IPA for isolation), resulting in the kafirin isolates Kaf_{EtOH} and Kaf_{IPA}, respectively. An extra defatting step with chloroform was required to increase the protein content of these isolates from $\approx 50\%$ (46–52%) to $\approx 80\%$ (79–84%). These chloroform-defatted Kaf_{EtOH} and Kaf_{IPA} differed in their functionality from each other. Kaf_{EtOH} remained as a coarse, sandy, paste-like dispersion when mixed at 55 °C with water or water containing 5% 2-mercaptoethanol. In contrast, Kaf_{IPA} aggregated into a cohesive mass in both, water and water containing 5% 2-mercaptoethanol. However, after this initial aggregation, the Kaf_{IPA} aggregate became immediately hard and brittle when only water was used, whereas it remained at least slightly extensible for a few minutes in water containing 5% 2-mercaptoethanol. The better performance of Kaf_{IPA} relative to Kaf_{EtOH} might be attributed to the higher hydrophobicity of 83% IPA relative to 70% ethanol. Kafirin is more hydrophobic than zein (Wall and Paulis, 1978) and therefore requires higher concentrations of organic solvents (e.g. acetonitrile) than zein for solubilization or extraction (Bean et al., 2000). Additionally, α-kafirins are more hydrophobic than β and γ kafirins, based on the solubility ranges reported by Shull et al. (1991). A more hydrophobic solvent would thus be expected to favor extraction of α-kafirins at the expense of β and γ-kafirins. RP-HPLC confirmed the presence of earlier-eluting (i.e. more hydrophilic) proteins in Kaf_{EtOH}, unlike in Kaf_{IPA}; however, the overall chromatograms obtained with these kafirins were not well resolved and showed broad elution areas, rather than sharp peaks (*data not shown*).

RP-HPLC analysis of kafirins has found that β-kafirins tend to have surface hydrophobicity similar to that of α-kafirins judging by their elution times (i.e. they elute near the same time as the α-kafirins) (Bean et al., 2000). Thus the use of the more hydrophobic IPA to extract kafirins may have resulted in less contamination from residual β-kafirins. It is interesting to note that β-zeins elute early in RP-HPLC separations of zein (e.g. Bean et al., 2000), suggesting that their surface hydrophobicity is much less than that of the α-zeins. Comparison of the predicted hydrophobicity of the β-kafirins and β-zeins from their sequence data also suggests that the β-kafirins are much more hydrophobic than the β-zeins. β-kafirins have a GRAVY score of 0.170 while β-zeins have a score of −0.091 (higher scores indicate a greater degree of hydrophobicity) indicating that the β-zeins are more hydrophilic, which matches with the RP-HPLC elution order of these proteins. For comparison, α-kafirins have a score of 0.193 and α-zeins 0.238 while γ-kafirins −0.227 and γ-zeins −0.367. These values match

the overall RP-HPLC elution order of the various subclasses. This may explain why extraction of zeins with 70% ethanol results in better separation of α-zeins from the β and γ zeins, but for sorghum, more non-polar solvents are needed to separate the α-kafirins from the β-kafirins. These numbers should be regarded with some caution as the hydrophobicity of kafirin subclasses is not clear. For example, measures such as hydration energy show that γ-kafirins are the most hydrophobic, yet they are water soluble (Belton et al., 2006) and elute first in RP-HPLC separations, suggesting at least that their surface hydrophobicity is the lowest (Bean et al., 2000). While RP-HPLC elution order may not be completely related to total hydrophobicity of a protein, surface hydrophobicity may be a better indicator in this instance as it would be important in protein–protein interactions.

More discussion is needed to understand the tendency of Kaf_{IPA} to rapidly become firm during aggregation experiments in warm water, as well as the slightly improving effect of 2-mercaptoethanol in these experiments. Some clarification was provided by the resolubilization of aged, aggregated kafirin (aged Kaf_{IPA}). Within the first hour in 60% *tert*-butanol plus 0.5% sodium acetate (without reducing agent), aged Kaf_{IPA} (present as suspended coarse particles from previous grinding) was not visibly solubilized. The subsequent addition of 2% 2-mercaptoethanol had some distinct effects: While within 5 min, some solubilization of the particles could be observed, and complete solubilization took 10 days. After addition of a second portion of 2-mercaptoethanol, within another 6 days, there still remained some insoluble small particles. This suggests that once aggregated, kafirin is very difficult to resolubilize. The need for reducing agent in addition to 60% *tert*-butanol indicates that hydrophobic interactions and disulfide bonds play a role. Even if the Kaf_{IPA} in this study should have been relatively pure α-kafirin (with only little remaining β and γ-kafirin), oligomers of kafirin have been reported to contain all subclasses of kafirins (El Nour et al., 1998).It is also noteworthy that Shull et al. (1991) used a reducing agent for the solubilization of α-kafirins. In fact, these authors defined α-kafirins by their solubility in 40–90% *tert*-butanol plus 2-mercaptoethanol, and it is possible that α-kafirins cross-linked to any β-kafirins may be more difficult to solublize due to the increased hydrophobicity of the β-kafirins or perhaps for some other unknown reason.

Structural similarities between zeins and kafirins have been reported (Belton et al., 2006). As such, in aggregation experiments with Kaf_{IPA} in water, a similar mechanism as suggested for zein aggregation can be assumed (i.e. formation of large aggregates with the most hydrophobic domains in the inside and the less hydrophobic moieties exposed to the surface). However, there may be a possible additional role of disulfide bonds. Some disulfide bonds may reform within the hydrophobic domains of the kafirin aggregates which may be inaccessible to 2-mercaptoethanol in water. This would explain why the aggregated Kaf_{IPA} quickly became very firm when placed in warm water, even if 2-mercaptoethanol was present. A very tight, cross-linked structure with disulfide bonds buried in hydrophobic domains would also explain that – even though very slowly - 60% *tert*-butanol plus 2-mercaptoethanol could largely dissolve aged Kaf_{IPA}. The presence of disulfide bonds that are inaccessible to reducing agent has also been suggested by Oria et al. (1995) and Duodu et al. (2002) to help explain the relatively low protein digestibility of cooked sorghum even in the presence of a reducing agent. Prediction of hydrophobic regions from known sequences of α, β, and γ kafirin (Kyte and Doolittle, 1982) revealed that some cysteine residues are located in predicted hydrophobic regions of the proteins. The single cysteine residue in the α-kafirin sequence used for these predictions was found in a hydrophobic region of the protein. For the β-kafirin sequence, 8 (out of 10) of the cys residues were found to be within

predicted hydrophobic regions of the protein. For the γ-kafirin sequence, 4 (out of 15) of the cys residues were found to be in predicted hydrophobic regions. Note that similar results were also found for α, β, and γ zein sequences.

Finally, the formation of non-disulfide cross-links (e.g. via oxidative coupling of tyrosine residues) has also been suggested in the context of low digestibility of cooked kafirin (Duodu et al., 2003). It cannot be excluded that such exotic bonds might play an additional role in the aggregation of kafirin in warm water into a firm and ultimately brittle substance.

4. Conclusions

When isolating zein from dry milled corn, the type of isolation procedure has a large influence on the ability of this zein to aggregate in warm water into a viscoelastic, gluten-like substance. In the present study, successful procedures isolated predominantly α-zein with less than ≈10% co-extracted β + γ zeins. This suggests that for zein aggregation, hydrophobic interactions play a central role, while disulfide bonds are undesirable. The best isolation procedure involved steeping under acidic and reducing conditions, followed by extraction with 70% aqueous ethanol. When extracting kafirin from dry milled sorghum, use of a more hydrophobic solvent (83% aqueous isopropanol) was required to produce kafirin with some functional properties. This kafirin aggregated in warm water, preferably with an added reducing agent, into a viscoelastic substance. However, such aggregated kafirin stiffened within minutes. These findings may be a first step in achieving gluten-like functionality of kafirin for breadmaking. However, more research is needed to understand inherent differences between zein and kafirin. From a practical point of view, it is critical to maintain kafirin's viscoelasticity in warm water for a longer time.

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