

Use of SDS to Extract Sorghum and Maize Proteins for Free Zone Capillary Electrophoresis (FZCE) Analysis¹

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ABSTRACT

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Two different extraction methods for extracting sorghum (*Sorghum bicolor* L. Moench.) storage proteins for free zone capillary electrophoresis (FZCE) analysis were compared. A traditional solvent based on 60% *t*-butanol was compared with a pH 10 borate buffer containing the anionic detergent SDS followed by precipitation of nonkafirins using 60% *t*-butanol. FZCE analysis of both types of extracts showed identical patterns, despite the fact that the SDS should have given all proteins equal charge-to-mass ratios. This methodology was also successfully applied to maize proteins. The use of *t*-butanol to precipitate nonkafirins, combined with electrophoresis at low pH, is thought to have removed the SDS from the

storage proteins. The SDS extraction procedure produced more stable extracts for FZCE analysis. These extracts could also be used directly for SDS capillary electrophoresis (SDS-CE) separations. Kafirins from 15 genotypes were extracted with this procedure and analyzed by FZCE and SDS-CE. Resolution of the kafirins by FZCE was much higher than the SDS-CE, demonstrating that the kafirin proteins possessed a high level of charge density variability within a relatively small molecular size distribution. Two distinct groups of α -kafirins could be seen in the FZCE electropherograms.

High-performance capillary electrophoresis (HPCE) has been used to separate proteins from all the major cereals: wheat, rice, barley, oats, maize, and sorghum (reviewed in Bean et al 1998; Bean and Lookhart 2000). HPCE is capable of separating proteins by differences in charge density in free zone capillary electrophoresis (FZCE), by differences in molecular mass (SDS-CE), or by differences in isoelectric point using capillary isoelectric focusing. There is also the possibility of separating proteins by differences in surface hydrophobicities using capillary electrochromatography (CEC) in a manner analogous to reversed-phase HPLC.

Two different FZCE buffers have been reported for separating sorghum storage proteins (Bean et al 2000). A buffer consisting of 80 mM phosphate-glycine containing 60% acetonitrile successfully differentiated several sorghum genotypes. However, some instability was reported using the traditional solvent based on 60% *t*-butanol (BuOH), especially of the γ -kafirins. Based on the work of Wallace et al (1990), Hamaker et al (1995) demonstrated that the use of an alkaline buffer containing the anionic detergent SDS and β -mercaptoethanol (β -ME) could effectively extract all proteins in sorghum (and maize). Nonkafirin proteins were then precipitated by the addition of organic solvents (Hamaker et al 1995). This procedure resulted in more efficient extractions of kafirin proteins.

This extraction procedure would seem useful for preparing samples for FZCE analysis. The anionic detergent SDS is widely used to produce size-based separations of proteins in SDS-PAGE and SDS-CE. This is possible, in part, because the SDS detergent binds all proteins roughly equal, producing uniform charge-to-mass ratios, and eliminates any differences in charge density. However, FZCE separates proteins based on differences in charge densities. Therefore, one would expect that the use of SDS to extract proteins

for FZCE would result in a complete loss of resolution. This report demonstrates that SDS can be used to prepare sorghum and maize proteins for FZCE analysis with improved sample stability. The same extracts can also be used for SDS-CE separations.

MATERIALS AND METHODS

Capillary Electrophoresis

All separations were made using either a Beckman PACE 2100 or PACE 5510 instrument. FZCE separations were made using 50 μ m i.d. \times 27 cm (20 cm L_d) uncoated fused silica capillaries (Polymicro, Phoenix, AZ). The FZCE buffer used was 80 mM phosphate-glycine containing 60% acetonitrile (ACN) plus 0.05% hydroxypropylmethyl-cellulose (HPMC) (2% = 4,000 cps). FZCE separation conditions were as described in Bean et al (2000). All samples were injected for 2 sec at 0.5 psi. Separations were monitored at 200 nm. Capillary rinsing protocols were as described in that study.

SDS-CE separations were performed using 75 μ m i.d. \times 27 cm (20 cm L_d) uncoated fused silica capillaries (Polymicro). The SDS-CE buffer was a commercial reagent (BioRad) modified by the addition of 15% ethylene glycol (Bean and Lookhart 1999). Separation and capillary rinsing protocols were as described in that study. All samples were injected for 15 sec at 0.5 psi.

Sample Preparation

The method of Hamaker et al (1995) was used to extract sorghum proteins for analysis. Briefly, whole sorghum and maize kernels were ground in a commercial coffee grinder for 30 sec. Ground samples (250 mg) were extracted at room temperature with 1 mL of a 12.5 mM sodium-borate buffer, pH 10, containing 1% SDS and 2% β -ME with continual vortexing using a vortex genie2 equipped with a 30-place vial holder (Fisher Scientific). Several different extraction times were investigated including 15, 30, and 60 min. This extraction procedure was repeated twice and 200 μ L of supernatant from each extraction was removed and pooled. To the pooled supernatant, 600 μ L of *t*-BuOH was added, and the samples were vortexed continually for 15 min, then centrifuged at 10,000 \times g for 5 min. The supernatants were then heated for 5 min at 98 to 100°C in a heating block. This heating step was added because the samples would be used in SDS-CE and heating of samples is routinely done before SDS-PAGE. This may also improve stability due to thermal denaturation of proteases. Aliquots were then removed for analysis.

In comparison to the modified Hamaker procedure, samples were also extracted with a solution of 12.5 mM sodium borate buffer, pH 10, containing 60% *t*-BuOH and 2% β -ME. To keep the

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sample volumes constant between the two types of extraction procedures, 600 μL of 12.5 mM sodium borate buffer, pH 10, containing 60% *t*-BuOH and 2% β -ME was added to 400 μL of protein extract when using this solvent. Likewise, these samples had the albumin and globulin proteins removed with preextraction as described in Bean et al (*in press*) because preextraction produced better results when using this solvent.

RESULTS AND DISCUSSION

When attempting to improve the extraction of sorghum storage proteins for FZCE, we found that the procedure of Hamaker et al (1995) using SDS produces FZCE separations that are identical to those not using SDS (Fig. 1). The same was found for maize storage proteins (Fig. 2). Obviously, the SDS must have been removed from the proteins when the alcohol was added to the extraction or at the beginning of the separation, or by a combination of the two.

Organic solvents such as methanol can disrupt SDS binding to proteins (Eckerson 1994) and the *t*-BuOH used during the extraction to precipitate the nonkafirins may also weaken the interaction between SDS and the proteins. The high level of ACN in the separation buffer may also help to weaken the SDS-protein interaction, especially because no SDS is present in the separation buffer. Decreasing the SDS-to-protein ratio can also affect the binding between SDS and proteins (Eckerson 1994). Thus, when injected into the capillary, an environment with no free SDS, the SDS-protein interaction could be weakened in a fashion similar to what occurs during protein blotting (Jungblut et al 1990; Eckerson 1994). Electroelution in SDS free buffers at low pH reportedly completely removes bound SDS from some proteins (Schuhmacher et al 1996), and SDS migrated off of proteins during isoelectric focusing in the presence of urea, which weakened the binding between SDS and proteins (Miller and Elgin 1974; Danno 1977).

Note that the samples extracted with the SDS procedure used by Hamaker et al (1995) produced excellent separation even though

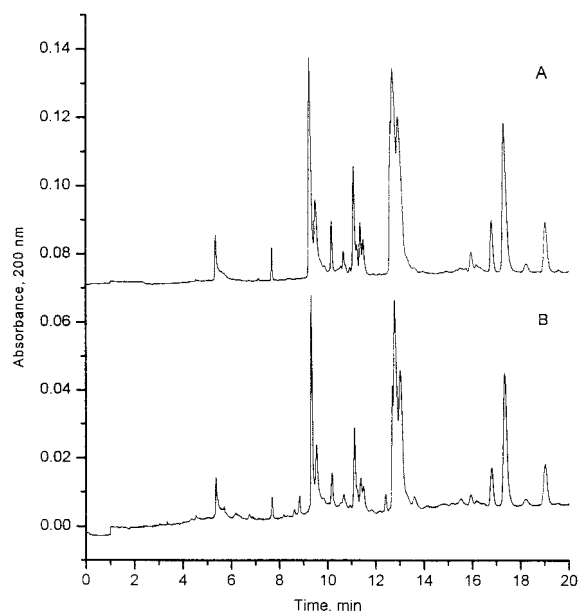


Fig. 1. Comparison of extraction solvents for sorghum kafirin proteins. **A**, Kafirins extracted with 60% *t*-butanol (BuOH) + 2% β -ME in a 12.5 mM borate buffer, pH 10.0, after removal of albumin and globulin proteins by selective extraction. **B**, Kafirins extracted with 1% SDS + 2% β -ME in a 12.5 mM borate buffer, pH 10.0, and nonkafirins precipitated by the addition of 60% *t*-BuOH. Samples were analyzed using a 50- μm i.d. uncoated fused silica capillary (27 cm long, 20 cm L_d) with 80 mM phosphate-glycine buffer, pH 2.5, containing 60% acetonitrile and 0.05% hydroxypropylmethyl-cellulose. Separation voltage 12.5 kV at 45°C. UV detection at 200 nm.

the samples were not preextracted to remove the albumin and globulin proteins. Previous work with maize and sorghum showed that good separations could not be obtained without first extracting with a combination of water and salt-containing buffers, especially for sorghum proteins (Bean et al 2000). However, in the procedure used by Hamaker et al (1995), nonkafirins and nonprotein nitrogen are precipitated after extraction. This precipitation step must have removed the compounds responsible for interfering with the FZCE analysis.

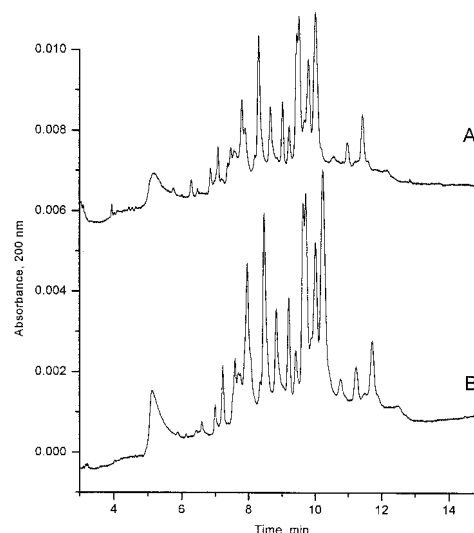


Fig. 2. Comparison of extraction solvents for maize zein proteins. **A**, Zeins extracted with 60% *t*-butanol (BuOH) + 2% β -ME in a 12.5 mM borate buffer, pH 10.0, after removal of albumin and globulin proteins by selective extraction. **B**, Zeins extracted with 1% SDS + 2% β -ME in a 12.5 mM borate buffer, pH 10.0, and nonkafirins precipitated by the addition of 60% *t*-BuOH. Samples were analyzed using a 50- μm i.d. uncoated fused silica capillary (27 cm long, 20 cm L_d) with 80 mM phosphate-glycine buffer, pH 2.5, containing 60% acetonitrile and 0.05% hydroxypropylmethyl-cellulose. Separation voltage 12.5 kV at 45°C. UV detection at 200 nm.

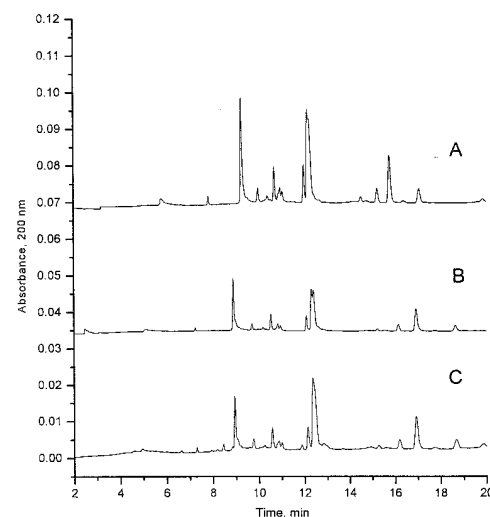


Fig. 3. Stability of kafirin samples extracted with SDS. **A**, Samples freshly extracted; **B**, after 24 hr of setting in high-performance capillary electrophoresis instrument; and **C**, sample stored in capped vial for three days. Kafirins extracted with 1% SDS + 2% β -ME in a 12.5 mM borate buffer, pH 10.0, and nonkafirins precipitated by the addition of 60% *t*-BuOH. Samples were analyzed using a 50- μm i.d. uncoated fused silica capillary (27 cm long, 20 cm L_d) with 80 mM phosphate-glycine buffer, pH 2.5, containing 60% acetonitrile and 0.05% hydroxypropylmethyl-cellulose. Separation voltage was 12.5 kV at 45°C. UV detection at 200 nm. Sample A analyzed on a different capillary than samples B and C.

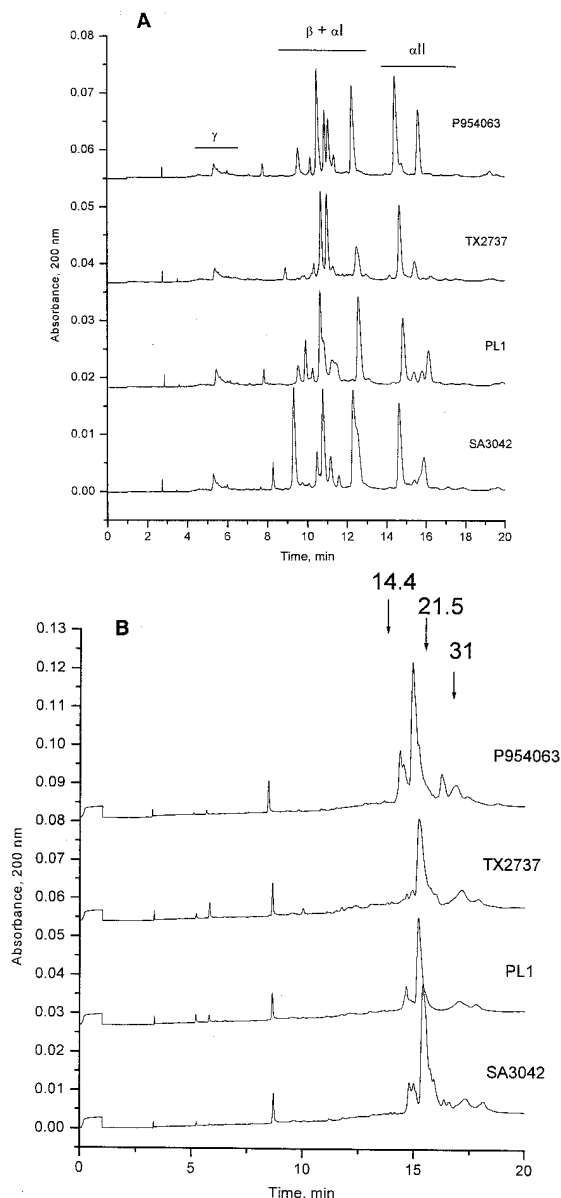


Fig. 4. Comparison of separation modes for four sorghum genotypes. **A**, Samples separated by free zone capillary electrophoresis as described in Fig. 1. **B**, Samples separated by SDS capillary electrophoresis using SDS-CE protein run buffer modified by the addition of 15% ethylene glycol. Arrows and numbers indicate approximate migration time of molecular weight standards. Aliquots of the same sample were used for both separation modes, demonstrating that the extraction protocol worked equally well for both types of separations.

Extracts prepared using the extraction procedure of Hamaker et al (1995) had good stability for at least 24 hr in the instrument and at least two to three days while kept in a capped vial (Fig. 3). We also found that the extraction and stability of the γ -kafirins and zeins were much improved and the repeatability of the extraction much higher (data not shown).

Extraction Time

Hamaker et al (1995) adopted their extraction protocol from one first reported by Wallace et al (1990), using two 1-hr extractions. Because this results in a rather lengthy total extraction time, we tested extraction times of 15, 30, and 60 min to see whether shorter extraction times could be used. Two extractions were made at each time. We found that at 30 min roughly the same amount of protein could be extracted as at 60 min, although the extraction-to-extraction repeatability was poorer with the shorter extraction

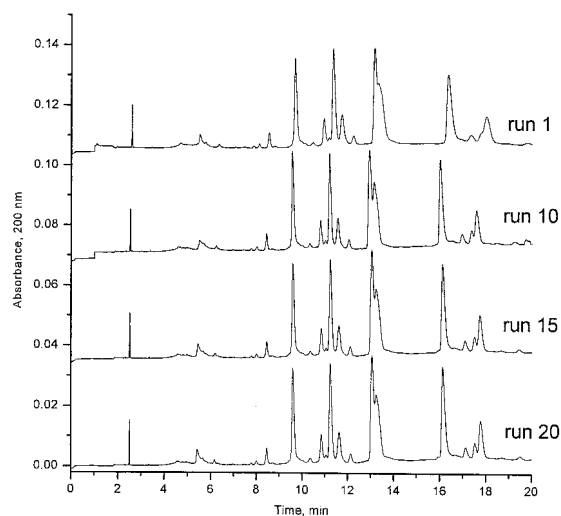


Fig. 5. Migration time repeatability of 15 consecutive injections. Kafirins extracted with 1% SDS + 2% β -ME in a 12.5 mM borate buffer, pH 10.0, and nonkafirins precipitated by the addition of 60% *t*-BuOH. Samples analyzed using a 50- μ m i.d. uncoated fused silica capillary (27 cm long, 20 cm L_d) with a 80 mM phosphate-glycine buffer, pH 2.5, containing 60% acetonitrile and 0.05% hydroxypropylmethyl-cellulose. Separation voltage 12.5 kV at 45°C. UV detection at 200 nm.

TABLE I
Peak Area Repeatability

Run Number	Kafirins Subclass (%)		
	γ	$\beta + \alpha$ -I	α -II
1	9.1	65.7	25.2
2	8.8	68.7	22.5
3	8.1	67.7	24.2
4	8.4	67.2	24.4
5	8.8	67.1	24.1
Average	8.6	67.3	24.1
Standard deviation	0.4	1.0	0.9
Relative standard deviations (%)	4.1	1.5	3.7

time (data not shown). Thus, two 1-hr extractions were used, the same as used by Hamaker et al (1995).

Separation Mode

In addition to FZCE, maize proteins have been separated by SDS-CE (Parris et al 1997), where proteins are separated primarily by differences in molecular mass. Kafirins from 15 genotypes and hybrids were extracted and analyzed to determine whether the SDS-based extracts could be directly analyzed by SDS-CE. These samples were also separated by FZCE for comparison. Separations of four genotypes are shown in Fig. 4. Good patterns were obtained with both separation techniques. These analyses were made on aliquots of the same samples; therefore, one extraction produced samples that worked equally well with either FZCE or SDS-CE.

Separating the samples by both techniques reveals interesting information about the proteins. The proteins had relatively narrow molecular weight distributions (Fig. 4B). However, these same proteins, when separated by charge, revealed large differences in charge density (Fig. 4A). Resolution of the FZCE separations was, therefore, much higher. The α -kafirins of sorghum reportedly are composed of two groups of polypeptides with apparent molecular masses of 23,000 and 25,000 Da, respectively (Shull et al 1991). FZCE separations clearly show many peaks in the α -kafirins regions (Bean et al 2000), suggesting that the α -kafirins are composed of many polypeptides or possibly protein isoforms. In fact, two distinct regions of α -kafirins can be seen: a group that comigrates with β -kafirins with a relatively high mobility, and a slower migrating group. We have designated these two groups α -I and α -II, respectively (Fig. 4A).

Repeatability

The repeatability of procedures used in this article were assessed by analyzing separate extractions and measuring the percent subclass composition and the total area. Migration time repeatability was checked by analyzing 15 consecutive injections. Separation-to-separation migration time repeatability was excellent (Fig. 5), with relative standard deviations (RSD) <0.3%, similar to that reported for maize using the same separation conditions (Bean et al 2000). Variability in capillary-to-capillary migration times were higher (RSD < 5%) and also similar to that reported for maize proteins. An example of capillary-to-capillary variability can be seen in Fig. 3 (compare electropherogram A to B and C). Peak area showed greater variability but repeatability was still high with RSD < 5% (Table I). We have found that the total area can vary considerably between capillaries. Attempts to standardize the areas with an internal marker, methyl green dye, were not successful (data not shown).

CONCLUSIONS

The extraction procedure of Hamaker et al (1995) can be successfully used to prepare sorghum and maize proteins for FZCE analysis despite the use of the anionic detergent SDS. Apparently, the SDS is removed from the proteins through the combination of organic solvent and low pH used to extract and analyze the proteins. This extraction procedure results in improved sample stability and does not require that the albumin and globulin proteins be preextracted. Improved extraction and stability was especially evident in the γ -kafirins.

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