

Glacial Acetic Acid—A Novel Food-Compatible Solvent for Kafirin Extraction

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There is increasing interest in industrial uses for kafirin, the prolamin protein of sorghum as an edible bioplastic film (Buffo et al 1997; Da Silva and Taylor 2005; Gao et al 2005) in applications such as fruit and nut coating (Enviropak 2004). Kafirin can be extracted from by-products of sorghum grain processing like bran (Da Silva and Taylor 2004). Kafirin is very similar in composition to zein (maize prolamin) but is slightly more hydrophobic (Duodu et al 2003). Effective extraction of kafirin requires the use of aqueous ethanol at elevated temperature (Johns and Brewster 1916) or aqueous tert-butanol (a higher alcohol) at ambient temperature (Taylor et al 1984), or extraction of total grain proteins in basic buffer containing sodium dodecyl sulfate (SDS) followed by precipitation of the nonkafirin proteins with aqueous tert-butanol (Hamaker et al 1995). There are problems with these processes for industrial and food use. Aqueous ethanol at elevated temperature is highly inflammable and not acceptable to certain religions. Tert-butanol is classified as not completely harmless in the Swiss poison class classification (Merck 2001).

Recently, we have shown that glacial acetic acid is a very effective ambient temperature solvent for kafirin and can be used for casting kafirin films (Taylor et al 2005). Glacial acetic acid appears to be effective on account of its low dielectric constant (6.1) (Merck 2001), which enables it to dissolve hydrophobic proteins such as kafirin. The objectives of this work were to determine whether glacial acetic acid is a good extractant for kafirin from sorghum grain and to compare its effectiveness with other food-compatible prolamin extractants, aqueous ethanol, and aqueous isopropanol (Lawton 2002).

MATERIALS AND METHODS

Sorghum

A mixture of two white, condensed tannin-free sorghum cultivars (PANNAR 202 and 606, from B. Koekemoer, Lichtenburg, South Africa, 2001) was used for kafirin extraction.

Extractants

Material used for kafirin extraction were 70% (w/w) ethanol containing 0.5% (w/w) sodium metabisulfite and 0.35% (w/w) NaOH at 70°C; 55% (w/w) isopropanol containing 0.5% (w/w) sodium metabisulfite and 0.35% (w/w) NaOH at 40°C; glacial acetic acid at 25°C; glacial acetic acid containing 0.5% (w/w) sodium metabisulfite at 25°C; glacial acetic acid after presoaking the flour for 1 hr or 16 hr with 0.5% (w/w) sodium metabisulfite at 25°C; glacial acetic acid after presoaking the flour for 16 hr with 1.0% (w/w) sodium metabisulfite at 25°C. After presoaking, the sodium metabisulfite solutions were removed by filtration under vacuum before extracting with glacial acetic acid.

The residual moisture content of the grain was $\approx 35\%$, reducing the final acetic acid concentration to $\approx 92\%$.

Kafirin Extraction

Kafirin was extracted from clean, whole grain milled using a laboratory hammer mill (Falling Number, Huddinge, Sweden) fitted with an 800- μm opening screen. Flour (500 g) was extracted for 1 hr with the given extractants (2.5 kg) at given temperatures with constant stirring. The extract was separated by centrifugation at $1,000 \times g$ for 5 min, and the alcohol solvents were allowed to evaporate from the solute overnight at ambient temperature from shallow open trays placed in a fume cupboard. The protein slurry was then washed with a minimal amount of cold ($<10^\circ\text{C}$) distilled water and the pH was adjusted to approximately pH 5 with 1M HCl to precipitate out the protein. The protein was recovered by filtration under vacuum and then freeze-dried.

When glacial acetic acid was used as an extractant, protein recovery was initially by precipitation on pH adjustment with saturated NaOH. This was an exothermic reaction heating the solution to 50°C . The resulting sodium acetate salt was then removed by washing several times with distilled water. In view of this high temperature, the extraction was repeated but the temperature was held at 25°C during pH adjustment by placing the extraction vessel in an ice water mixture. In subsequent work, the glacial acetic acid was removed by dialysis against distilled water (10°C) with two changes. The protein was recovered as above. The kafirin preparations were defatted with hexane at ambient temperature at a protein-to-solvent ratio of 1:10 (w/w).

Protein Determination

Protein ($N \times 6.25$) was determined using the Dumas total combustion method. Protein yields for the extractions were calculated as weight of total recovered protein divided by the grain protein content $\times 100$.

Characterization of Kafirin Preparations

Amino acid composition was determined using the Pico-Tag method (Bidlingmeyer et al 1984) by reverse-phase HPLC after precolumn derivatization. SDS-PAGE under reducing and non-reducing conditions was conducted as described by Da Silva and Taylor (2004). Well loading was at constant protein ($\approx 10 \mu\text{g}$).

Statistical Analysis

Extraction experiments were repeated at least twice. Analysis of variance using the least squares procedure was applied.

RESULTS AND DISCUSSION

Table I shows that the aqueous ethanol and aqueous isopropanol-based metabisulfite-containing solutions were effective extractants at elevated temperature for kafirin, with a recovery of 75–80% with a single extraction based on the estimate of sorghum grain kafirin content of 68–73% made by Hamaker et al (1995). Glacial acetic acid at ambient temperature, even with added metabisulfite, was not effective. The amount of protein extracted was less than half that of the other two solvents. However, when the grain was presoaked in metabisulfite, as in maize wet-milling (Jackson and

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Shandera 1995), the yield of protein (presumed kafirin) was greatly increased. In fact, if the grain was presoaked 16 hr in 0.5–1.0% metabisulfite, the recovery of kafirin was 80–90%. Steeping of maize in sulfur dioxide cleaves disulfide bonds and so disrupts intermolecular aggregates in native zein (Boundy et al 1967). It is suggested that presoaking the sorghum grain in sulfur dioxide before extraction with glacial acetic acid would act similarly on the sorghum matrix proteins, causing their partial disruption. In water, sulfite anions, bisulfite anions, and sulfur dioxide (as a dissolved gas) exist in equilibrium based on sulphurous acid (Jackson and Shandera 1995). Boundy et al (1967) suggested that sulfurous acid in solution would react with cystine to produce soluble S-sulfocysteine residues within the maize intercellular protein matrix. Jackson and Shandera (1995) thought that the formation of S-sulfocysteine would prevent the reformation of disulfide bonds and so partially break down the matrix structure.

Concerning the purity of the proteins extracted, that extracted with glacial acetic acid with metabisulfite presoak was very much less pure than those extracted with the aqueous ethanol or aqueous isopropanol-based solvents (Table II). However, after washing (desalting) the glacial acetic acid preparation, the purity was increased to the same concentration as those from the other two solvents. It is presumed that adjustment of the glacial acetic acid protein extract to pH 5 with NaOH resulted in the formation of sodium acetate salt, which was responsible for the low purity of the preparation. Subsequently this problem was avoided by dialysing the glacial acetic acid protein extract against distilled water to remove the glacial acetic acid and precipitate out the kafirin. After defatting the kafirin preparations with hexane, purities were all the same, ≈90%. The amount of lipid co-extracted with the kafirin was high, probably because whole grain was used.

The amino acid compositions of the three kafirin preparations were essentially identical, being rich in glutamine (measured as glutamic acid), leucine, and alanine, and very low in lysine (Table III). Their compositions were virtually identical to that of kafirin, as reported by Taylor and Schüssler (1986).

The SDS-PAGE band patterns (both reducing and nonreducing) of the protein preparations from the three different extractants were essentially the same (Fig. 1). They are typical of kafirin, as reported by El Nour et al (1998), showing kafirin monomers $\alpha 1$, $\alpha 2$, β , and γ kafirin dimers and oligomers. However, the $\alpha 1$, $\alpha 2$, and γ kafirin monomer apparent M_r values were ≈4 k lower than

that reported by El Nour et al (1998), possibly as a result of using different molecular weight markers.

SDS-PAGE under nonreducing conditions (Fig. 1B) showed some differences between the glacial acetic acid-extracted kafirin preparations, depending on their method of recovery. Kafirin recovered by pH adjustment with no temperature control (lane 4) showed far more intense dimers (arrow) and more intense oligomers, and far less intense monomers compared with the other glacial acetic acid extracts and the aqueous alcohol extracts. The monomers presumably polymerized into dimers and oligomers as a result of exposure to the moist, high temperature from pH adjustment, in the same manner as when sorghum is wet-cooked (Duodu et al 2002).

The kafirin preparations from extraction with glacial acetic acid and recovered by pH adjustment with temperature control to 25°C (lane 4) or by dialysis (lane 3) under nonreducing conditions (Fig. 1B) showed a band of M_r 28–30 × 10³. Also, only very faint bands of the β -kafirin monomers (M_r 20, 18, and 16 × 10³) were present in all three glacial acetic acid extracts. However, under reducing conditions, definite β -kafirin monomer bands were present in these preparations (Fig. 1A). Thus, it is possible that the M_r 28–30 × 10³ band was a disulfide bond-linked dimer of β -kafirin, although no reference could be found to such a dimer in the literature. El Nour et al (1998) observed that β -kafirin was only found in its monomeric form except when very rigorous extraction conditions were used. Then it was present in polymers with $\alpha 1$ - and γ -kafirin.

It is concluded that in combination with a presoaking step using a reducing agent such as sodium metabisulfite, glacial acetic acid can be very effective for extracting kafirin from sorghum grain. Also, as early work showed that glacial acetic acid is a good zein solvent (reviewed by Lawton 2002), it is probable that the above-described process would also be suitable for extracting zein from maize. Further, in view of the potential advantages of using glacial acetic acid, such as its volatility and no requirement for alcohol licence nor religious concerns (unlike ethanol), it may have industrial as well as laboratory application. The only proviso is that it is essential that the solvent temperature is kept low to prevent polymerization of the prolamin molecules.

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TABLE I
Effect of Different Extractants on Yield and Recovery of Kafirin^a

Extractant	Yield (% of total grain protein)	Recovery of Kafirin (%) ^b
70% ethanol + 0.5% sodium metabisulfite + 0.35% NaOH at 70°C	54.3b (3.0)	74.4–79.8
55% isopropanol + 0.5% sodium metabisulfite + 0.35% NaOH at 40°C	55.3bc (0.2)	75.8–81.3
Glacial acetic acid at 25°C	25.0a (1.0)	34.2–36.8
Glacial acetic acid + 0.5% sodium metabisulfite at 25°C	25.0a (1.4)	34.2–36.8
Presoak (1 hr) 0.5% sodium metabisulfite, glacial acetic acid at 25°C	52.8b (9.2)	72.3–77.6
Presoak (16 hr) 0.5% sodium metabisulfite, glacial acetic acid at 25°C	59.3cd (1.3)	81.2–87.2
Presoak (16 hr) 1.0% sodium metabisulfite, glacial acetic acid at 25°C	61.0d (0.3)	83.6–89.7

^a Values followed by the same letter in the same column are not significantly different ($P > 0.05$). Figures in parentheses indicate standard deviations.

^b Values calculated according to estimates of the kafirin content of sorghum grain (68–73%) made by Hamaker et al (1995).

TABLE II
Effect of Different Extractants on Purity of Extracted Kafirin

Extractant	Purity (% dwb) ^a	
	Before Defatting	After Defatting
70% ethanol + 0.5% sodium metabisulfite + 0.35% NaOH	74.6b (2.5)	89.3a (2.8)
55% isopropanol + 0.5% sodium metabisulfite + 0.35% NaOH	73.1b (1.9)	91.2a (1.9)
Presoak (16 hr) 0.5% sodium metabisulfite, glacial acetic acid ^b	42.8a (14.9) (before washing salt out)	
	68.0b (1.9) (after washing salt out)	92.9a (0.5) (after washing salt out)

^a Values followed by the same letter in the same column are not significantly different ($P > 0.05$). Figures in parentheses indicate standard deviations.

^b Temperature held at 25°C during pH adjustment.

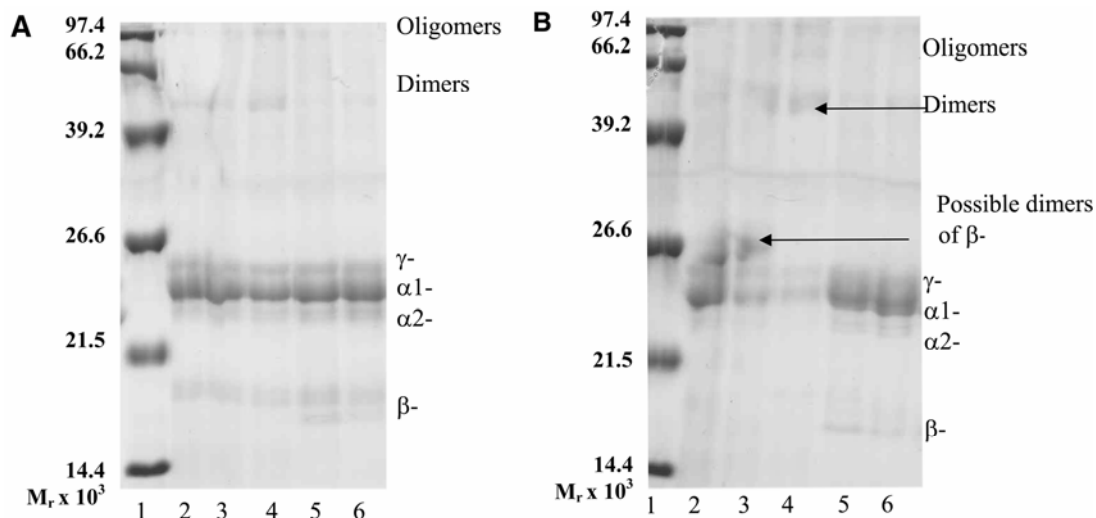


Fig. 1. SDS-PAGE of kafirin extracted with different extractants. **A**, reducing conditions; **B**, non-reducing conditions. 1 = Molecular weight standards; 2 = glacial acetic acid, removed by dialysis; 3 = glacial acetic acid, protein recovered by pH adjustment, held at 25°C; 4 = glacial acetic acid, protein recovered by pH adjustment, no temperature control; 5 = 55% isopropanol-based; 6 = 70% ethanol-based.

TABLE III
Amino Acid Composition (g/16 g of N) of Kafirin Extracted with Different Extractants

Amino Acid	70% Ethanol + 0.5% Na Metabisulfite + 0.35% NaOH (70°C)	55% Isopropanol + 0.5% Na Metabisulfite + 0.35% NaOH (40°C)	Presoak 0.5% Na Metabisulfite, Glacial Acetic Acid (25°C)	60% Tert Butanol + 0.05% Dithiothreitol (25°C) ^a
Aspartic acid ^b	5.5	5.6	6.0	5.2
Glutamic acid ^b	24.6	23.0	24.4	22.0
Serine	5.3	5.1	5.1	3.9
Glycine	1.9	2.1	2.1	1.7
Histidine	1.7	1.5	1.6	2.2
Arginine	1.8	2.2	2.8	1.8
Threonine	3.1	3.1	3.1	2.8
Alanine	10.6	10.2	10.7	10.6
Proline	9.7	8.9	9.0	9.8
Tyrosine	5.0	4.8	4.0	4.9
Valine	4.3	4.2	4.8	4.8
Methionine	1.6	1.6	0.5	2.4
Isoleucine	3.7	3.6	4.1	4.3
Leucine	16.4	15.3	15.7	14.7
Phenylalanine	5.7	5.6	5.5	5.8
Lysine	0.5	0.7	0.7	0.2

^a From Taylor and Schüssler (1986).

^b Mainly in the amide form.

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