Efficient Procedure for Extracting Maize and Sorghum Kernel Proteins Reveals Higher Prolamin Contents Than the Conventional Method[1]

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ABSTRACT

A new method to separate maize proteins into zein (prolamin) and nonzein classes was used to quantitatively measure the prolamins (zein and kafirin) and nonprolamins in five maize and sorghum genotypes. Some 90–95% of protein was extracted using a pH 10 buffer containing 1% sodium dodecyl sulfate and 2% 2-mercaptoethanol. Ethanol or *t*-butanol was added to the maize and sorghum extracts at 70 or 60%, respectively, to precipitate nonprolamin proteins; those remaining in solution were prolamins. The amount of protein in these fractions was compared to that obtained with the conventional Landry-Moureaux method which uses aqueous alcohol plus 0.6% 2-mercaptoethanol as the extraction solvent. Using this new method, zein contents of 50–60% of total protein were found for maize whole grain flours, with 62–74% zein for endosperm alone. Kafirin contents were 68–73% in sorghum whole grain flours, and 77–82% in sorghum endosperm. Prolamin and nonprolamin fractions were judged to be pure based on SDS-PAGE. Zein contents determined by the new method were between 5–12% higher than those obtained by the conventional procedure; kafirin contents were 12–22% higher. This new method provides a highly reproducible, accurate procedure for determining the content of prolamin and nonprolamin proteins. Yet it is simpler and faster than the conventional fractionation method.

Zeins and kafirins, the prolamin storage proteins of maize and sorghum grains, respectively, are soluble in aqueous alcohol plus a reducing agent. There are many similarities between the prolamins of the two cereals. Both are found in protein bodies in the endosperm and are structurally related. They have been subclassified as α-, β-, and γ-zeins or -kafirins based on similarities in molecular weight, solubility, and structure (Esen 1987, Shull et al 1991). There also is a minor low M_r fraction (M_r 9,000 to 10,000) in maize designated as δ-zein that has not yet been described for sorghum. Within subclasses, antibodies cross-react between zeins and kafirins (Shull et al 1991), and a high degree of sequence homology has been shown between γ-zein and γ-kafirin (de Barros et al 1991). α-Prolamin is the major storage protein in both cereals and is found in the central part of the protein body. β- and γ-Prolamins are found either at the protein body periphery or in dark-staining inclusions within protein bodies (Lending and Larkins 1989, Shull et al 1992). In maize, α-zein comprises 75–85% of the total zein and is made up of two groups of related polypeptides of M_r 22,000 and 19,000 (Larkins et al 1989). Sorghum α-kafirin contains two groups of polypeptides of M_r 25,000 and 23,000 (Shull et al 1991). These proteins comprise 66–71% and 80–84% of the total kafirin in the opaque and vitreous kernel sections, respectively (Watterson et al 1993). β-Zein makes up 10–15% of maize prolamin and a methionine-rich polypeptide of M_r 14,000; β-kafirin comprises 7–8% of sorghum prolamin and has three polypeptides of M_r 20,000, 18,000, and 16,000. γ-Zein, which comprises 5–10% of the maize prolamin in normal genotypes, contains a M_r 27,000 and a M_r 16,000 protein, both of which are proline-rich polypeptides; γ-kafirin comprises 9–12% of the total kafirin and consists of a polypeptide of M_r 28,000 (Esen 1987, Larkins et al 1989, Shull et al 1991, Watterson et al 1993).

Prolamins were first identified by Osborne (1924) as proteins extracted in aqueous alcohol, after removal of the water and salt soluble proteins. Later, large amounts of prolamin were extracted when a reducing agent was included in the aqueous alcoholic solvent. Landry and Moureaux (1970) incorporated the use of reducing agents into their much used fractionation scheme for maize zeins. A further modification of this procedure was developed for extraction and quantitation of sorghum kafirins (Wall and Paulis 1978).

The Landry-Moureaux method divides prolamins into two major classes: those extractable in aqueous alcohol alone and those extractable in aqueous alcohol plus a reducing agent. These classes have been termed zein or zein-I, and zein-like or zein-II (also called G_1 or alcohol-soluble glutelins). Another minor fraction, termed prolamin-like (G_2 or salt-soluble glutelins) (Landry et al 1983), contains a prolamin-like protein that may be the γ-zein described by Esen (1987). This fraction is obtained from the alcohol-extracted pellet using a solution containing salt and reducing agent at pH 10. Total prolamin content has frequently been estimated as that obtained by adding the two classes (zein and zein-like) together or by extraction with aqueous alcohol plus reducing agent (Taylor et al 1984b, Wilson 1987, Watterson et al 1993).

In maize, total zein content (Landry and Moureaux fractions II plus III) was reported as 52% of the protein content in whole grain and 60% in endosperm (Wilson 1987). Misra and Mertz (1975) found 65% zein in endosperm of the maize inbred Oh43. Sorghum kafirin is known to be less extractable than maize zein (Wall and Paulis 1978). Jambunathan and Mertz (1973), using 70% isopropanol with and without 0.6% 2-mercaptoethanol (2-ME) to extract prolamins sequentially, found total kafirin in two normal (intermediate hardness, low lysine), low tannin sorghum cultivars to be 37.3 and 28.8% (of total protein) for the whole grain, and 55.0 and 43.3% in endosperm. Guiragossian et al (1978), who also extracted with isopropanol, reported 50.2% kafirin in the endosperm of the sorghum cultivar P721 N (normal, low tannin type). Paulis and Wall (1979) used 60% *t*-butanol to extract kafirins and 60% *t*-butanol plus 2-ME to extract what they termed alcohol-soluble reduced glutelins. Later Taylor et al (1984b) performed an extensive study of the conditions and solvents used in fractionation of sorghum proteins to obtain the

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maximum extraction of the various protein classes. They found that 60% *t*-butanol plus reducing agent was a superior solvent for extracting kafirins at room temperature, compared to 70% isopropanol plus reducing agent. The mean kafirin content of 41 whole grain sorghum cultivars was 47.8% (of total protein) with a range of 37.5–59.2%.

Wallace et al (1990) described a simpler method to separate maize kernel proteins into zein (subclassified as the α-, β-, γ-, and δ-zeins) and nonzein fractions. This method is based on extraction of virtually all the kernel proteins in borate buffer (pH 10) with a detergent (1% SDS) and reducing agent (2% 2-ME), followed by addition of alcohol to 70% total volume. This last step precipitates nonzein proteins, while zeins remain in solution. This procedure provides clear separation of maize endosperm proteins into two types, with no evidence of cross contamination as shown by SDS-PAGE and immunoblotting analysis. Wallace et al (1990) used this procedure to extract total zein, but reported only the relative values of α-, β-, and γ-zein using ELISA. In the present study, we used this method to measure the prolamin and nonprolamin proteins in maize and sorghum seeds, and compared these values to those obtained using the conventional extraction method of Landry-Moureaux.

MATERIALS AND METHODS

Sample Preparation

The maize (W64A+, Oh43, Mo17, B73, and W64*o2*) and sorghum genotypes (P721 N, Sepon 82, SRN 39, SC283-14, and P721 Q) used in the study were grown in 1993 at the Purdue Agronomy Farm, West Lafayette, IN. Samples were ground using a Tecator Cyclotec mill (model 1093, Sweden) to whole grain flour through a 0.5-mm mesh screen, or were treated as follows. Maize kernels were hand-degermed using an adjustable speed drill (Dremel, Racine, WI). Sorghum grain was decorticated and hand-degermed to remove any remaining germ. These endosperm samples were ground to flour as described above. Whole grain flours were defatted using petroleum ether. Moisture contents of the flours were determined by the oven drying method (AACC 1983).

Protein Fractionation Methods

Whole grain and endosperm proteins were fractionated into prolamins and nonprolamins similar to the method described by Wallace et al (1990). Samples (200 mg) were extracted for 1 hr on a shaker at room temperature with 0.0125*M* sodium borate, 1% SDS, 2% 2-ME (pH 10.0) at a ratio of 1:10 (flour-solvent). The suspension was centrifuged at 5,000 × *g* for 20 min and the supernatant was saved. The procedure was repeated twice and the supernatants were pooled. For maize, ethanol was added to a final concentration of 70%, while for sorghum *t*-butanol was added to a final concentration of 60%. The mixture was allowed to stand for 2 hr with occasional stirring, then centrifuged at room temperature. The supernatant contained the prolamin (zein or kafirin) and nonprotein nitrogen fractions and the pellet contained detergent-extractable nonzeins or nonkafirins. To determine true prolamin content, nonprotein nitrogen, along with albumin and globulin proteins, was first removed from the flour sample by extraction with 0.5*M* NaCl at 4°C three times followed by two water washes. After each extraction, suspensions were centrifuged and supernatants discarded. Proteins in the resulting pellets were then extracted and fractionated as described above according to the Wallace et al (1990) procedure and prolamin content determined. The above protein fractions (prolamin plus

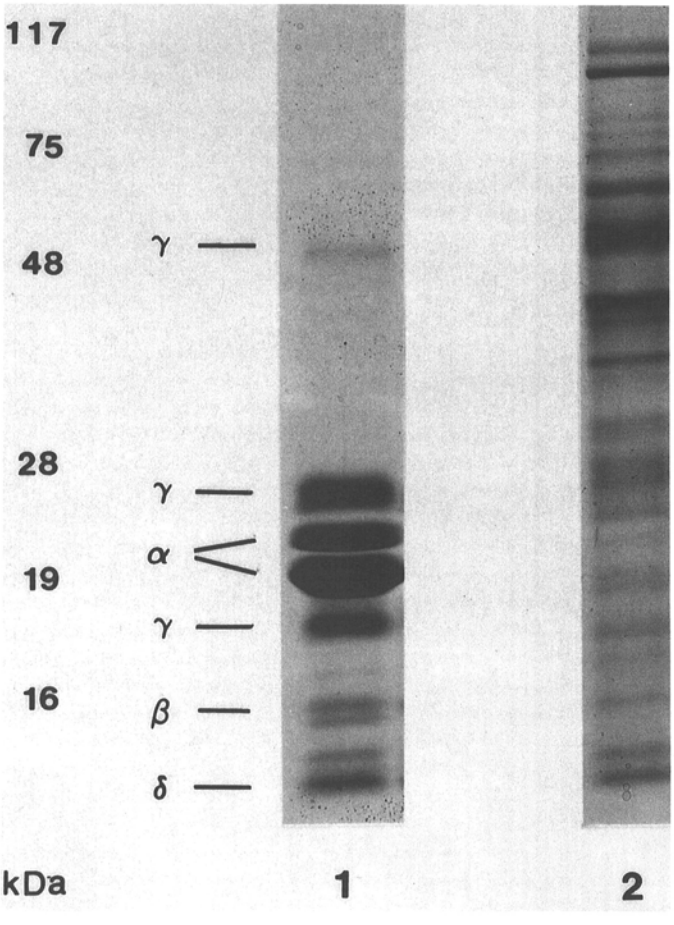


Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of zein (lane 1) and nonzein (lane 2) fractions of maize endosperm (genotype W64 A+) obtained from the fractionation method of Wallace et al (1990).

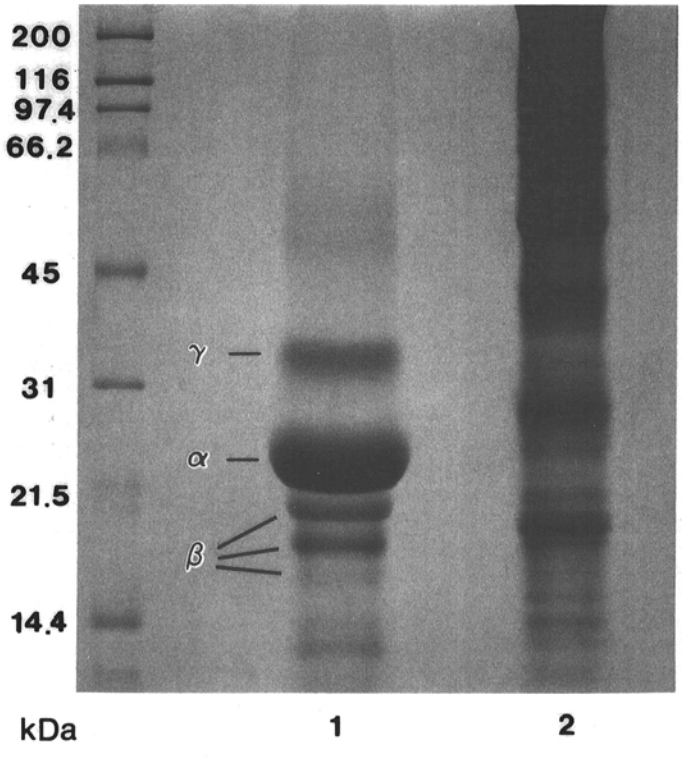


Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of kafirin (lane 1) and nonkafirin (lane 2) fractions of sorghum endosperm (genotype P721 N) obtained from the fractionation method of Wallace et al (1990).

nonprotein nitrogen, prolamin, and nonprolamin) were placed in microKjeldahl tubes, dried at 80°C, and analyzed for nitrogen by the microKjeldahl method (AACC 1983). Total nitrogen was also determined on flour samples and converted to protein (N × 5.7) (Mosse 1990). Preliminary testing showed that SDS did not interfere with nitrogen determination. Samples were analyzed in triplicate.

Prolamin content was also determined for maize and sorghum whole grain and endosperm flours using the method of Landry-Moureaux (1970) for maize and a modified procedure (Taylor et al 1984b) for sorghum. Flour samples were extracted with 0.5*M* NaCl three times at 4°C for 60, 30, and 30 min, centrifuged, and supernatants discarded. The pellet was then washed with water twice for 30 min and supernatants discarded. Zein was obtained using 70% 2-propanol plus 0.6% 2-ME; kafirin was obtained using 60% *t*-butanol with 0.6% 2-ME. Pellets were extracted three times using the scheme described above; supernatants were pooled, dried as described above; nitrogen was determined by microKjeldahl as described above.

SDS-PAGE

Protein fractions were obtained by the Wallace et al (1990) method from maize (W64A+) and sorghum (P721 N) endosperm flour. The prolamin-containing supernatants were dialyzed extensively against water at 4°C and lyophilized. The nonprolamin-containing pellets were washed with the appropriate alcoholic solution (70% ethanol or 60% *t*-butanol) then lyophilized. SDS-PAGE was performed on a vertical gel electrophoresis system (BRL model V15.17, Gaithersburg, MD). The separating gel was a linear 10–15% (w/v) polyacrylamide gradient containing 5*M* urea with a 4% (w/v) stacking gel. Lyophilized samples were dissolved in SDS-PAGE sample solvent (2% SDS, 1% 2-ME, 0.066*M* Tris at pH 6.8, with 10% glycerol and bromophenol blue), heated in a boiling water bath for 3 min, and placed in the sample wells. A broad range molecular weight standard (6,500–200,000) was used (BioRad, Richmond, CA). Electrophoresis was performed at 70 V for 18 hr. Proteins were stained with 0.25% Coomassie Brilliant Blue R-250 in 46% methanol and 8% acetic acid and destained in 20% ethanol and 10% acetic acid.

Statistics

Significant differences between mean prolamin values obtained from the two methods were determined using Student's *t*-test. One-way analysis of variance and the Student-Newman-Keuls sequential range test were used to determine statistical differences ($P < 0.05$) between group means. Precision of the method was measured in five replicate samples as: coefficient of variation = standard deviation/mean × 100 (Steel and Torrie 1980).

RESULTS AND DISCUSSION

Methodology

The method described by Wallace et al (1990) was highly repeatable and accurate for obtaining prolamin and detergent-extractable nonprolamin contents of maize and sorghum flour. The coefficient of variation for true zein contents of the five whole grain maize genotypes ranged from 1.4 to 4.5%. For true kafirin contents of the five whole grain sorghum genotypes, the coefficient of variation ranged from 0.1 to 1.1%. The purity of the prolamin and nonprolamin fractions was tested by SDS-PAGE (Figs. 1 and 2). Banding patterns of each protein fraction were visually inspected for contamination by the other fraction. Maize zein (Fig. 1, lane 1) and sorghum kafirin (Fig. 2, lane 1) formed the typical banding patterns described in previous reports (Taylor et al 1984a, Hamaker et al 1986, Wallace et al 1990) as α, ß, γ, and δ for maize (Esen 1987, Wallace et al 1990) and α, ß, and γ for sorghum (Shull et al 1991). These fractions were essentially free of contamination by nonprolamins. Conversely, the nonprolamin fractions of both cereals did not show notable contamination by the prolamin proteins (Figs. 1 and 2, lane 2). In developing this new method for maize protein fractionation, Wallace et al (1990) went further in examining the purity of the separation by showing that antibodies against α-, β-, and γ-zein proteins did not react with proteins in the nonzein fraction. This method appears to function equally well in fractionating sorghum proteins, since there was also no apparent cross-contamination of the kafirin and nonkafirin fractions.

Prolamin and Nonprolamin Contents

True zein contents of normal maize genotypes (Oh43, Mo17, W64 A+, B73) ranged from 50.4 to 56.2% in the whole grain, and 62.2 to 73.9% in the endosperm (Table I). True zein (and kafirin) was obtained by first removing the nonprotein nitrogen (with albumin and globulin proteins), followed by extraction of proteins in the borate buffer with SDS plus 2-ME and precipitation of nonprolamins with alcohol. The *opaque-2* maize mutant (W64 *o2*) showed substantially lower zein content and higher nonzein content as expected. Nonzein contents were proportionally higher in whole grain flour when compared to the endosperm due to the presence of the germ, which is high in

TABLE I
Maize Protein Fractionated into Zein and Nonzein Using the Wallace Method[a]

Genotype	Protein[b]	Zein + NPN[c]	Zein (True)[d]	Nonzein	NPN[e]	Nonextractable[e]
Whole grain						
Oh43	11.5 b[f]	71.3 a	59.7 a	23.8 b	11.6	4.9
Mo17	10.2 c	73.1 a	54.8 b	22.5 b	18.3	4.4
W64 A+	13.6 a	67.1 a	56.2 b	21.2 b	10.9	11.7
B73	9.5 c	68.7 a	50.4 c	28.0 b	18.3	3.3
W64 *o2*	12.3 b	59.3 b	33.9 d	35.9 a	25.4	4.8
Endosperm						
Oh43	10.5 b	79.7 a	73.9 a	16.7 b	5.8	3.6
Mo17	9.5 c	74.7 b	69.1 b	19.4 b	5.6	5.9
W64 A+	12.6 a	73.4 b,c	69.0 b	18.9 b	4.4	7.7
B73	9.0 c	70.8 c	62.2 c	22.7 b	8.6	6.5
W64 *o2*	10.5 b	62.4 d	47.9 d	31.0 a	14.5	6.6

[a] Wallace et al (1990).
[b] Percent of flour, dry weight basis.
[c] Alcohol-soluble portion extracted in pH 10 buffer with sodium dodecyl sulfate (SDS) plus 2-ME.
[d] Above portion extracted following initial NaCl extraction to remove NPN.
[e] Nonprotein nitrogen (NPN) and nonextractable protein calculated by difference.
[f] Mean values within a column for each group that do not have a common letter are significantly different at $P < 0.05$.

TABLE II
Sorghum Protein Fractionated into Kafirin and Nonkafirin Using the Wallace Method[a]

Genotype	Protein[b]	Kafirin + NPN[c]	Kafirin (True)[d]	Nonkafirin	NPN[e]	Nonextractable[e]
Whole Grain						
SC283-14	10.7 d[f]	79.1 e	68.1 b	14.6 a	11.0	6.3
P721 N	9.8 e	87.5 b	68.4 b	5.9 d	19.1	6.6
Sepon 82	10.9 b	84.1 c	68.3 b	7.8 c	15.8	8.1
SRN 39	11.8 a	89.3 a	72.9 a	5.3 e	16.4	5.4
P721 Q	10.7 c	82.4 d	63.8 c	8.9 b	18.6	8.7
Endosperm						
SC283-14	9.0 d	88.0 a	82.0 a	7.7 b	6.0	4.3
P721 N	8.9 d	86.0 a	80.1 a,b	6.1 d	5.9	7.9
Sepon 82	10.1 b	86.8 a	77.6 b	7.0 c	9.2	6.2
SRN 39	10.8 a	86.8 a	77.1 b	5.9 d	9.7	7.3
P721 Q	9.3 c	83.1 b	72.4 c	10.3 a	10.7	6.6

[a] Wallace et al (1990).
[b] Percent of flour, dry weight basis.
[c] Alcohol-soluble portion extracted in pH 10 buffer with sodium dodecyl sulfate (SDS) plus 2-ME.
[d] Above portion extracted following initial NaCl extraction to remove NPN.
[e] Nonprotein nitrogen (NPN) and nonextractable protein calculated by difference.
[f] Mean values within a column for each group that do not have a common letter are significantly different at $P < 0.05$.

nonprolamin protein. Nonzein contents for normal maize genotypes ranged from 21.2 to 28.0% in the whole grain and 16.7 to 22.7% in the endosperm. Nonprotein nitrogen values (calculated) were higher than those reported by Wilson (1987); they were similarly higher in whole grain than endosperm, the average for the normal genotypes was 14.8% for whole grain and 6.1% for endosperm. The *opaque-2* mutant had a surprisingly high nonprotein nitrogen content. Christianson et al (1965) showed that over 50% of nonprotein nitrogen is free amino acids. The mean calculated nonextractable protein was relatively low, 5.9%, indicating that nearly all protein and nonprotein nitrogen was extracted in the initial solvent. Recoveries ranged from 88.3 to 96.7%.

Prolamin content in the sorghum genotypes was higher than that in maize. True kafirin contents for normal sorghum genotypes (SC283-14, P721 N, Sepon 82, SRN 39) ranged from 68.1 to 72.9% for whole grain, and 77.1 to 82.0% for endosperm (Table II). Nonkafirin contents were correspondingly lower, ranging from 5.3 to 14.6% in the whole grain samples and 5.9 to 7.7% in the endosperm. The high-lysine sorghum mutant (P721 Q) had slightly but significantly lower kafirin content and higher nonkafirin content (in the endosperm) compared to the normal genotypes (Table II). Nonprotein nitrogen was again higher in the whole grain samples and lower in the endosperm samples. Nonextractable protein was low and recoveries ranged from 91.3 to 95.7%.

Comparison of the Landry-Moureaux and Wallace Methods

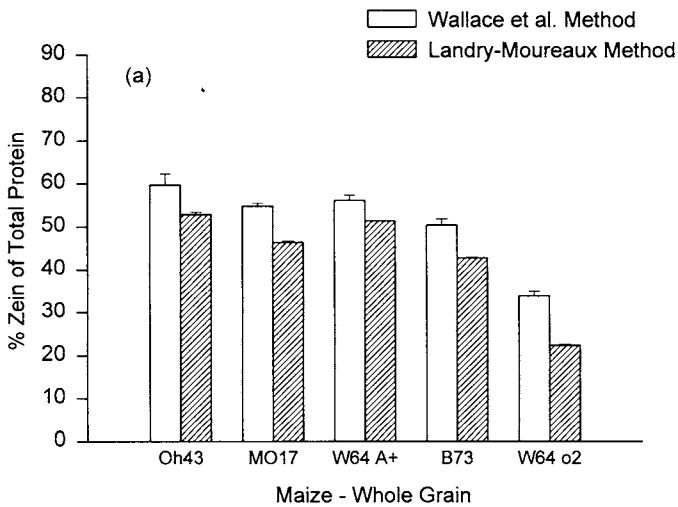
In all cases, prolamin contents determined by the method of Wallace et al (1990) were higher than those obtained using the conventional Landry-Moureaux procedure (Figs. 3 and 4). Differences between the methods for the recovery of prolamins were, in general, larger for sorghum compared to maize. In whole grain maize samples, the difference between the methods ranged from 4.8% for W64 A+ to 11.5% for W64 *o2*. In maize endosperm samples, the range was similarly 4.5% for W64 A+ to 10.6% for W64 *o2*. Quantitation of zein was most different in the *opaque-2* mutant (Fig. 3). Wallace et al (1990) demonstrated using Western blot analysis that their method completely separated zeins from nonzeins, while zein proteins could still be detected in the Landry-Moureaux combined fractions IV and V (G_2 and G_3 glutelins). Zeins were more extractable in the new method because they were initially all extracted in the Landry-Moureaux glutelin extraction buffer, and then separated from the nonzeins. The difference between the two methods could be accounted for by Landry-Moureaux fraction IV (G_2 glutelin), which contains some of the γ-zein protein (Esen 1987, Landry et al 1983). However, it is also possible that some zein is retained in the true glutelin fraction (Landry-Moureaux fraction V or G_3 glutelin).

For the five sorghum genotypes tested, kafirin contents measured by the Wallace method were substantially higher than those determined with the Landry-Moureaux procedure (Fig. 4); differences were 18.0–22.4% for whole grain and 11.8–20.1% for endosperm. The kafirin values obtained with the new method were also markedly higher than literature values (Taylor et al 1984b, Hamaker et al 1986). Kafirin contents obtained from the Landry-Moureaux method were 49.0–54.7% for whole grain from normal genotypes and 59.6–70.2% for endosperm. This rather large and significant difference between the two methods attests to the known difficulty in extracting sorghum kafirins and supports the value of this new fractionation method to quantitatively measure kafirins.

CONCLUSIONS

This study shows that the Wallace et al (1990) method for extraction and quantitation of maize and sorghum prolamin and nonprolamin proteins is a useful and, in some respects, a superior procedure to those previously described. The advantage of this method lies in its simplicity and its apparently more accurate measurement of the prolamin class. The new procedure facilitates identification of zeins and kafirins as α-, β-, γ-, and (in the case of maize) δ-prolamins on the basis of differences in molecular weight, solubility, and structure. It allows zeins or kafirins to be extracted in one group. Further measurement of these proteins can be done by antibody (ELISA) or other tests. This method circumvents the rather complicated and cumbersome classification scheme whereby maize and sorghum prolamins are classified strictly on their extractability characteristics that places them into three or more solubility fractions: zein, zein-like, and prolamin-like (Landry and Moureaux 1970). Zein and zein-like fractions have been shown to overlap, and consequently, zein-like (G_1 glutelins) have been further divided into two subgroups: water-soluble alcohol-soluble and water-insoluble alcohol-soluble reduced glutelins (Landry et al 1983). While there is good reason to define and characterize proteins within solubility classes, using the new classification scheme (α, β, γ, δ), there is no apparent reason to subdivide the prolamins on the basis of solubility differences.

Another advantage of this new method is that it provides a rapid way of quantitating the nonprolamin proteins. It has been difficult to accurately categorize these proteins as albumins, globulins, and glutelins due to the shift in extractability of these



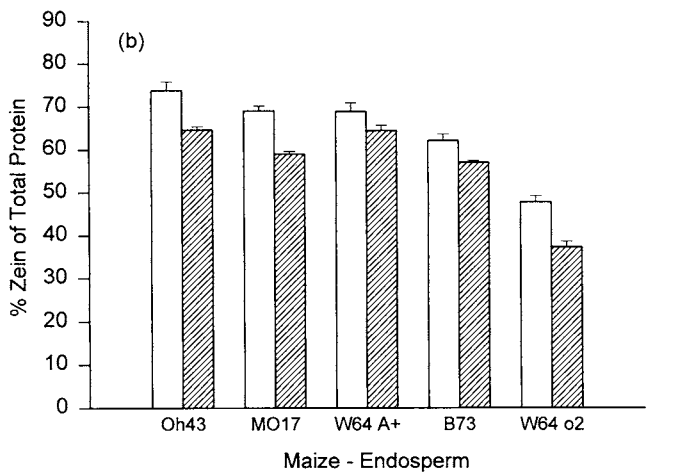
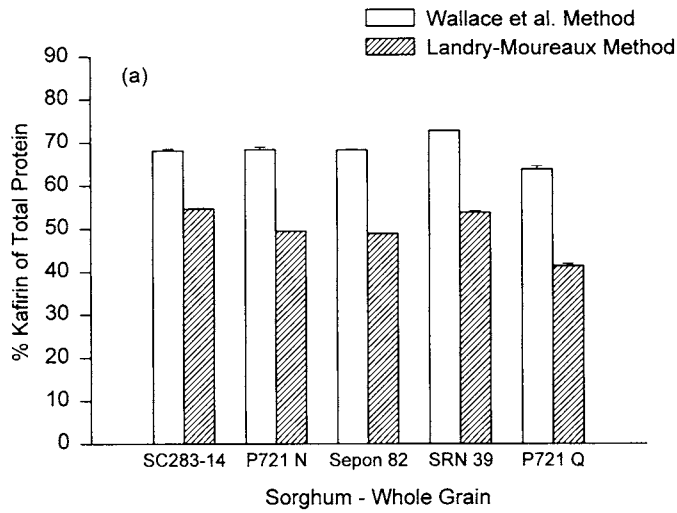


Fig. 3. A comparison of zein contents of five maize genotypes from whole grain (a) and endosperm (b) as determined by the method of Wallace et al (1990) and the conventional Landry-Moureaux method. Error bars indicate standard deviations. In all cases, differences between the two methods were significant at $P < 0.05$.



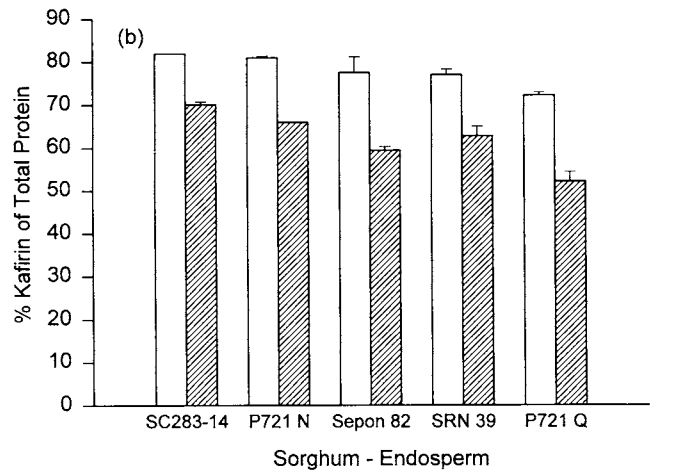


Fig. 4. A comparison of kafirin contents of five sorghum genotypes from whole grain (a) and endosperm (b) as determined by the method of Wallace et al (1990) and the conventional Landry-Moureaux method. Error bars indicate standard deviations. In all cases differences between the two methods were significant at $P < 0.05$.

proteins during seed development. It is perhaps easier to quantitate them as one group from which the individual proteins can then be characterized or isolated using other techniques. Thus, the clean separation of the two fractions coupled with the near complete extraction of the prolamin proteins in the SDS plus 2-ME buffer indicates that this method can be used in the future to assess prolamin and nonprolamin contents of maize and sorghum grain.

The much higher values for kafirin we describe may change the way we think about sorghum protein in terms of its functional and nutritional properties. When humans consume sorghum grain it is commonly decorticated, resulting in flours with kafirin content of ≈70–80% rather than 40–60% of total protein. Since kafirin is found in protein bodies that remain intact during cooking, this protein does not functionally interact with the other grain components. Thus, in light of the present data, only ≈10–20% of the protein, the nonkafirins (including nonextractable protein), may have functional properties important for making the flat breads, porridges, couscous, etc., that are eaten in Africa, Asia, and Latin America. In contrast, wheat gluten, which contains prolamin and glutelin protein, does not contain intact protein bodies. Both types of proteins are free to interact with other components in the flour to generate doughs.

Nutritionally, kafirin is of poor quality, because it contains practically no lysine and little tryptophan and threonine (Guiragossian et al 1978). It is also the least digestible protein in sorghum (Bach Knudsen and Munck 1985, Hamaker et al 1986). The high level of kafirin reported here provides some insight as to why sorghum grain has inferior nutritional quality and digestibility compared to that of most other cereals.

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