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2001

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Hicks, C.; Bean, S. R.; Lookhart, G. L.; Pedersen, Jeffrey F.; Kofoid, K. D.; and Tuinstra, M. R., "Genetic Analysis of Kafirins and Their Phenotypic Correlations with Feed Quality Traits, In Vitro Digestibility, and Seed Weight in Grain Sorghum" (2001). *Agronomy & Horticulture -- Faculty Publications*. 929.  
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# Genetic Analysis of Kafirins and Their Phenotypic Correlations with Feed Quality Traits, In Vitro Digestibility, and Seed Weight in Grain Sorghum<sup>1</sup>

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## ABSTRACT

Cereal Chem. 78(4):412–416

Twenty-three entries of grain sorghum (*Sorghum bicolor* (L.) Moench), including eight inbred lines (five males and three females) and 15 hybrids, were evaluated to determine the proportion of  $\gamma$ ,  $\alpha$ II, and  $\beta$ - $\alpha$ I-kafirins and their association with contents of crude protein, fat, and starch; protein digestibility; in vitro dry matter disappearance; and seed weight. The male lines included three normal-seeded lines (TX2737, TX435, and P954063) and two large-seeded lines (Eastin1 and PL-1). Female lines consisted of three common U.S. seed parent lines (Wheatland, Redlan, and SA3042). The lines and their hybrids were grown under

dryland conditions at two locations in Kansas using a randomized complete block design. The effects of genotype, location, and males were significant for all kafirins. Wide variations in composition and general combining ability (GCA) for kafirin content were noted among parent lines and hybrids, with TX2737, Eastin1, and PL1 having the largest GCA values for  $\gamma$  (1.37),  $\alpha$ II (1.99), and  $\beta$ - $\alpha$ I (2.57), respectively. Correlations among kafirins ranged from -0.89 to 0, whereas those of kafirins with feed quality traits, digestibility, and seed weight ranged from -0.45 to 0.48.

Sorghum is the second most important feed grain in the United States cattle feedlot industry. Apart from being a major source of dietary energy, it is also a major source of dietary protein. The kafirins, storage proteins found in protein bodies, are the most abundant, making up between 70 and 80% of the total endosperm protein in sorghum (Watterson et al 1993; Hamaker et al 1995). Kafirins have been classified as  $\alpha$ ,  $\beta$ , and  $\gamma$  according to differences in molecular weight, solubility, and structure (Esen 1987; Shull et al 1991). Several studies have investigated the qualitative and quantitative differences and distribution of kafirins in the kernel and endosperm (Shull et al 1991, 1992; Watterson et al 1993; Bean et al 2001). Protein quality is associated with the quantity and composition of protein fractions in the grain, and variation in genotype may affect these parameters (Hamaker et al 1995). Moreover, digestibility is an important attribute of high quality protein. To the extent that kafirins constitute the bulk of total protein, genetic improvement of protein content and quality would require an understanding of the quantitative variation and distribution of kafirins with respect to genotype and their relationship with feed quality traits and digestibility.

Recently, sorghum researchers in the United States and Australia have focused plant breeding efforts on developing large-seeded sorghum hybrids with improved yield potential and feed quality. In an analysis of genetically diverse sorghum lines and hybrids, Hicks et al (unpublished data) reported significant variation in seed size and combining ability for feed quality traits and in vitro digestibility. However, the biochemical basis for this variation is not well understood. Reports by Hamaker et al (1995) and Oria et al (2000) suggested that protein structure primarily determines variation in digestibility. However, variation in seed size and other grain characteristics also may affect kafirin composition and, thus, the relationships with feed quality traits and diges-

tibility. Therefore, the objectives of this study were to 1) investigate genetic variation and combining ability for kafirin composition and 2) determine the associations of kafirin composition with major feed quality traits, in vitro digestibility, and seed weight in sorghum cultivars and hybrids.

## MATERIALS AND METHODS

Hicks et al (unpublished data) evaluated 23 sorghum lines and hybrids for feed quality and agronomic characteristics. Given the cost associated with protein analysis, a subset of grain samples from these experiments was evaluated for kafirin composition. Five male and three female parent lines were intercrossed using a Design-II mating scheme to produce 15 hybrids (Comstock and Robinson 1952; Hallauer and Miranda 1988). The males used in this study included three normal-seeded lines (TX2737, TX435, and P954063) and two large-seeded lines (PL-1 and Eastin1). The females in this study were common U.S. seed parent lines (Wheatland, Redlan, and SA3042). The lines and their hybrids (23 entries) were planted and grown under dryland conditions at Kansas State University experiment fields in Ashland and Belleville, KS. The planting dates at the two locations were 27 and 28 May 1999, respectively. The experiment design for the original field trials consisted of a randomized complete block design with four replicates at each location. Entries were grown in single-row plots that were  $\approx 6$  m long and 0.76 m apart. Plots were thinned by hand to ensure uniform populations of 129,000 plants  $\text{ha}^{-1}$ . Samples of grain from individual plots were cleaned, dried, and ground through a 1-mm screen in a Udy mill.

Ground samples were evaluated for contents of crude protein (CP), crude fat (FAT), and crude starch (STA); protein digestibility (PD); and in vitro dry matter disappearance (IVDMD) as described by Hicks et al (unpublished data). In summary, CP was quantified based on total nitrogen measured using the nitrogen combustion method (CN-2000, Leco Corp., St. Joseph, MI). The CP in each sample was estimated from total nitrogen ( $\text{N} \times 6.25$ ) (Mosse 1990). The FAT was extracted and quantified using the ether extraction method 920.39 (AOAC 1990). The STA was evaluated similarly, using method 979.10. PD was quantified using a modification of the pepsin digestion procedure described by Mertz et al (1984). Ground flour samples were incubated in the buffered pepsin enzyme solution at 37°C for 2 hr in a shaking water bath. PD (g/kg) was calculated by subtracting undigested protein from total protein and was expressed as a proportion of total protein. The IVDMD was quantified using the procedure developed by Pedersen et al (2000). Ground samples were sealed in ANKOM F57 filter bags and were placed in vessels containing buffer solution and rumen

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inoculum. The samples were incubated at 39°C for 12 hr. IVDMD (g/kg) was calculated as the difference between the initial sample and residue sample weight and was expressed as a proportion of the initial sample.

Kafirin proteins were extracted and quantified from 92 grain samples representing two complete replicates of each entry at each location. The protein samples were evaluated for  $\gamma$ ,  $\alpha$ II, and  $\beta$ - $\alpha$ I kafirins using the procedure developed by Bean et al (2001). As described by Bean et al (2000), these three classes of sorghum storage proteins correspond precisely with the classical  $\alpha$ -,  $\beta$ -, and  $\gamma$ -kafirin designations of sorghum storage proteins; the only difference lies in the procedure for quantifying the components. In summary, ground samples were placed in an extraction solution containing 12.5 mM sodium-borate buffer, pH 10, with 1% SDS and 2%  $\beta$ -ME. Nonkafirins were precipitated by the addition of 60% t-BuOH. The solutions containing kafirins were separated and characterized by free-zone capillary electrophoresis (FZCE) using a Beckman Pace 5510 instrument. The FZCE separations were made using uncoated fused silica capillaries (50  $\mu$ m, i.d.  $\times$  27 cm; Polymicro, Phoenix, AZ) and 80 mM phosphate-glycine containing 60% acetonitrile (ACN) plus 0.05% hydroxypropylmethyl-cellulose (HPMC) (Bean et al 2001). All samples were injected for 2 sec at 0.5 p.s.i. Separations were monitored at 200 nm.

Analyses of variance (ANOVA) for kafirin composition were conducted based on two replicates of data at each location using the PROC GLM procedure of the Statistical Analysis System software package (SAS Institute, Cary, NC). The preliminary analysis involved tests for the effects of location, entry, and location-entry interaction. Expected mean squares for each source of variation were computed and used to apply the appropriate *F* test according to standard procedures (Hallauer and Miranda 1988). Entries were partitioned into variation within parents and within hybrids. The parent sum of squares was subdivided into males and females. Hybrid sums of squares were partitioned into male, female, and male-female interaction. Tests of significance for entry main effects and partitioned entry effects for all traits were made by testing the mean squares with the respective location interaction mean squares. Simple correlations based on plot means were computed to determine the associations among the kafirins. Correlations between kafirins and feed quality traits, in vitro digestibility, and seed weight also were computed based on plot means from the original analysis described by Hicks et al (unpublished data). Combining ability effects for each parent were computed using the methods of Beil and Atkins (1967). The general combining ability (GCA) value for an individual was determined by calculating the difference between the average performance of its hybrids and the performance of all hybrids evaluated in the test. Therefore, positive GCA values indicate that those lines produce hybrids with higher than average kafirin content for that particular fraction. The significance of GCA effects was determined using the least square significance difference as proposed by Cox and Frey (1984). The specific combining ability (SCA) effects were evaluated using the male-female interaction.

## RESULTS

The mean squares from analyses of variance for  $\gamma$ ,  $\alpha$ II, and  $\beta$ - $\alpha$ I kafirins are presented in Table I. The effects of location and entry were highly significant for each of the kafirin types; however, the effect of location-entry interaction was not significant. The entry source of variation was partitioned to evaluate the significance of variation among different subgroups of entries. Significant differences among inbred lines were noted for all kafirin types. The effect of hybrid was significant for  $\alpha$ II and  $\beta$ - $\alpha$ I kafirins, but not for  $\gamma$ . No significant differences between inbred and hybrid performance occurred for any of the kafirin types. The hybrid source of variation was partitioned to determine the importance of the male and female parent in expression of each kafirin. The effect

of male parent was significant for all types, but the effect of the female parent was significant only for  $\beta$ - $\alpha$ I kafirins. A significant effect of male-female interaction was noted for  $\alpha$ II and  $\beta$ - $\alpha$ I. Very little location interaction was observed except for the location-female interaction for  $\gamma$ . The values for coefficient of determination were 0.71–0.81. The values for coefficient of variation were 3.38–16.43%, with  $\gamma$  and  $\beta$ - $\alpha$ I kafirins being the most and least variable types, respectively.

The phenotypic correlations among the kafirins, feed quality traits, protein digestibility, and seed weight are presented in Table II. Significant negative correlations were detected between  $\beta$ - $\alpha$ I and  $\gamma$  (–0.44) and  $\beta$ - $\alpha$ I and  $\alpha$ II (–0.89). Significant correlations also were noted between CP and each of the kafirin types; those with  $\gamma$  (–0.21) and  $\alpha$ II (–0.23) were negative, and that with  $\beta$ - $\alpha$ I (0.30) was positive. PD showed a significant positive correlation with  $\gamma$  (0.48), but a significant negative correlation with  $\beta$ - $\alpha$ I (–0.45). The correlation between  $\alpha$ II and PD was not significant. All other correlations were low and not significant, except for a positive relationship between  $\alpha$  II and STA (0.23).

Least square mean values for kafirin content are presented in Table III. Wide variation in kafirin contents was noted among parental lines and hybrids. Lines or hybrids with high contents of one kafirin type did not necessarily have high contents of the other groups. The contents of  $\gamma$  were 6.38–10.74% of total kafirin among the parental lines and 7.12–10.38% for the hybrids, with Eastin1 $\times$ Wheatland and TX2737 $\times$ Redlan having the smallest and largest contents, respectively, among the hybrids. The contents of

**TABLE I**  
Means Square Values<sup>a</sup> from Analyses of Variance  
to Characterize Kafirin Types in Grain Sorghum Cultivars  
and Their F1 Hybrids Tested Over Two Locations

Source of Variation <sup>b</sup>	df	$\gamma$ -kafirin	$\alpha$ II-kafirin	$\beta$ - $\alpha$ I-kafirin
Location (L)	1	36.19**	59.60*	188.67**
Replicate (per L)	2	6.48*	0.42	8.14
Entry (E)	22	5.00**	29.50**	36.53**
Inbred (I)	7	9.15**	36.55*	59.84**
I vs. H	1	2.02	33.42	51.84
Hybrid (H)	14	3.14	25.70**	23.78**
Male (M)	4	7.75*	24.24**	36.79**
Female (F)	2	3.89	99.02	64.31**
M $\times$ F	8	0.65	8.10*	7.13*
L $\times$ E	22	1.38	4.12	4.00
L $\times$ I	7	1.12	5.45	7.06
L $\times$ I vs. H	1	0.24	15.11	19.12
L $\times$ H	14	1.59	2.68	1.38
L $\times$ M	4	0.74	1.50	0.84
L $\times$ F	2	5.05*	6.22	0.14
L $\times$ M $\times$ F	8	1.14	2.38	1.97
Error a <sup>1</sup>	28	1.48	3.73	3.76
Error b <sup>2</sup>	44	1.76	6.21	5.49

<sup>a</sup> \* and \*\* = significant at *P* < 0.05 and 0.01, respectively.

<sup>b</sup> Error a<sup>1</sup> = error term for design II analysis and Error b<sup>2</sup> = general error term. *R*<sup>2</sup> and CV = coefficients of determination and variation, respectively.

**TABLE II**  
Phenotypic Correlations Among Types of Kafirins, Feed-Quality  
Characteristics, Protein Digestibility, and Seed Weight  
of Grain Sorghum<sup>a</sup>

Trait <sup>b</sup>	$\gamma$ -kafirin	$\alpha$ II-kafirin	$\beta$ - $\alpha$ I-kafirin
$\alpha$ II	0.00	...	...
$\beta$ - $\alpha$ I	–0.44**	–0.89**	...
CP	–0.21*	–0.23*	0.30**
PD	0.48**	0.26	–0.45**
IVDMD	0.10	–0.05	0.00
FAT	0.08	–0.15	0.10
STA	–0.04	0.23*	–0.19
SW	–0.06	–0.11	–0.07

<sup>a</sup> \* and \*\* = significant at *P* < 0.05 and 0.01, respectively.

<sup>b</sup> CP = crude protein, PD = protein digestibility, IVDMD = in vitro dry matter disappearance, FAT = crude fat, STA = crude starch, and SW = seed weight.

$\alpha$ II were 17.78–27.04% for the parental lines and 18.15% in PL1 $\times$ Redlan to 25.66% in TX2737 $\times$ SA3042 for the hybrids. The contents of  $\beta$ - $\alpha$ I also varied, at 65.01–75.10% for the parental lines and from as low as 65.39% in TX2737 $\times$ SA3042 to as high as 74.06% in PL1 $\times$ Redlan for the hybrids.

GCA is the average performance of the progeny of an individual in a series of crosses with other genotypes. Significant positive and negative GCA values were observed for the lines evaluated in this test. The values of GCA for  $\gamma$ ,  $\alpha$ II, and  $\beta$ - $\alpha$ I are presented in Table IV. GCA effects varied markedly among the males and females, ranging from highly significant negative values to highly significant positive values. Male parent GCA effects were generally larger and more significant than female parent effects. TX2737 had the largest GCA value for  $\gamma$  (1.37), and Eastin1 and PL-1 had the largest GCA values for  $\alpha$ II (1.99) and  $\beta$ - $\alpha$ I (2.57), respectively. Among the females, only GCA effects for  $\beta$ - $\alpha$ I were significant, with Redlan expressing the highest effect.

The complex inheritance of kafirins in TX2737, Redlan, and TX2737 $\times$ Redlan is shown in Fig. 1. Individual kafirin types were identified based on absorbance peaks obtained during FZCE analy-

sis. FZCE analysis indicates that each kafirin type consists of many different protein subunits. Although inheritance studies indicated significant combining ability effects for both male and female parents, in this example the F1 hybrid expressed a protein profile much more similar to the female parent Redlan than the male parent TX2737. The hybrid pattern was not simply a composite of the two parental protein profiles, suggesting that factors other than gene numbers determine relative expression of storage proteins.

## DISCUSSION

The observations of quantitative variation in proportions of kafirins and their relationship with feed quality traits, in vitro digestibility, and seed weight reported in this study are unique. These relationships provide new insights about the complexity of the kafirin protein family and how they relate to feed characteristics. The study highlights some of the potential problems and challenges that may be encountered by breeders trying to breed for increased protein content and digestibility (Bramel-Cox et al 1990). The three kafirin types,  $\alpha$ II,  $\beta$ - $\alpha$ I, and  $\gamma$ , observed in this study are in agreement with our earlier report (Bean et al 2001), although they slightly depart from the traditional  $\alpha$ -,  $\beta$ -, and  $\gamma$ -kafirins reported in most of the literature (Watterson et al 1993). The  $\alpha$ II,  $\beta$ - $\alpha$ I, and  $\gamma$  kafirin designations used in this study correspond precisely with the classical descriptions for  $\alpha$ -,  $\beta$ -, and  $\gamma$  kafirins (Bean et al 2000). The difference between these results and those previously reported is not in content but in method of protein analysis, which vary as discussed by Bean et al (2000).

TABLE III  
Least Square Mean Values for Kafirin Content  
of Grain for Sorghum Parent Lines and Hybrids

	$\gamma$ -kafirin	$\alpha$ II-kafirin	$\beta$ - $\alpha$ I-kafirin
<b>Males</b>			
Eastin1	7.95	27.04	65.01
P954063	7.86	23.61	68.53
PL1	9.41	23.75	66.84
TX2737	10.74	22.06	67.20
TX435	6.38	20.96	72.65
<b>Females</b>			
Redlan	7.05	18.36	74.59
SA3042	6.46	20.99	72.55
Wheatland	7.13	17.78	75.10
<b>Hybrids</b>			
Eastin1 $\times$ Redlan	8.43	24.43	67.15
Eastin1 $\times$ SA3042	8.20	25.33	66.48
Eastin1 $\times$ Wheatland	7.12	25.46	67.41
P954063 $\times$ Redlan	8.36	22.24	69.40
P954063 $\times$ SA3042	8.06	24.32	67.61
P954063 $\times$ Wheatland	7.76	24.49	67.75
PL1 $\times$ Redlan	7.79	18.15	74.06
PL1 $\times$ SA3042	7.34	23.73	68.95
PL1 $\times$ Wheatland	7.18	21.93	70.90
TX2737 $\times$ Redlan	10.38	18.28	71.35
TX2737 $\times$ SA3042	8.95	25.66	65.39
TX2737 $\times$ Wheatland	9.34	25.06	65.61
TX435 $\times$ Redlan	8.51	19.58	71.91
TX435 $\times$ SA3042	7.26	24.06	68.68
TX435 $\times$ Wheatland	8.10	23.58	68.32
Standard error	0.66	1.25	1.17
LSD <sup>a</sup>	1.89	3.55	3.34

<sup>a</sup> Least significant difference ( $P < 0.05$ ).

TABLE IV  
General Combining Ability of Male and Female Parents  
for Kafirin Content of Sorghum Grain

	$\gamma$ -kafirin	$\alpha$ II-kafirin	$\beta$ - $\alpha$ I-kafirin
<b>Males</b>			
Eastin1	-0.27**	1.99**	-1.72**
P954063	-0.12	0.60	-0.48**
PL1	-0.76**	-1.82**	2.57**
TX2737	1.37**	-0.09	-1.28**
TX435	-0.23	-0.68**	0.91**
Standard error	0.22	0.32	0.24
<b>Females</b>			
Redlan	0.51	-2.55	2.04**
SA3042	-0.22	1.54	-1.31**
Wheatland	-0.28	1.02	-0.73**
Standard error	0.41	0.46	0.07
Grand mean	8.19	23.08	68.73

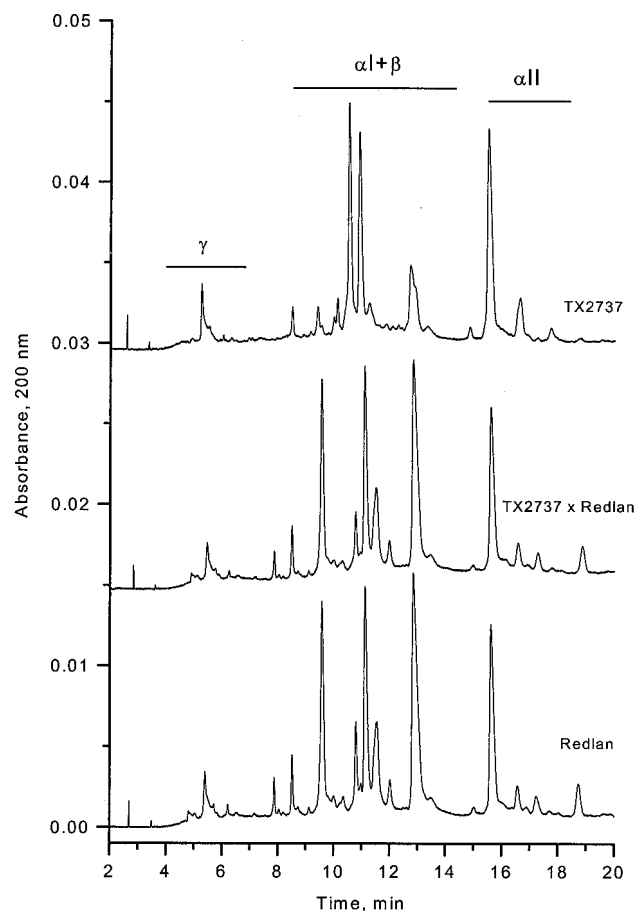


Fig. 1. Peaks obtained during kafirin extraction using free-zone capillary electrophoresis, indicating inheritance of kafirins in grain sorghum from the male parent (TX2737) and female parent (Redlan) to the F1 hybrid (TX2737  $\times$  Redlan).

The considerable variation among parent lines and hybrids in the proportions of kafirins is of significant interest. These results reinforce the argument that kafirin is a heterogeneous group of proteins differing in proportion and distribution with respect to genotype (Taylor et al 1984a,b; Sastry et al 1986). The quantitative variation in the distribution of kafirins observed in this study is understandable, given the diversity of the genotypes used. The parent lines differed significantly in kafirin contents. An analysis of the inheritance of kafirin protein patterns in hybrids indicated both additive and dominant genetic inheritance patterns. This is consistent with the results reported earlier by Sastry et al (1986). However, the unequal contribution of parental lines to hybrid performance as indicated by differences in GCA suggests that care should be taken when selecting the parent lines to produce hybrids with the desired combination of traits. Additionally, significant male-female interactions for  $\alpha$ II and  $\beta$ - $\alpha$ I indicate the importance of nonadditive inheritance and of evaluating both hybrids and parent lines for these protein fractions.

Numerous factors may affect the content of kafirins in grain. In this study, both genotype and location had significant effects. The significant effect of location and lack of location-entry interaction suggest that, although the phenotypic performance of the genotypes differed with respect to location, the differences in performance did not affect the ranking of the genotypes in the two environments. The effect of location on the kafirins may be explained by differences in soil nutrients and weather conditions typical between the two locations. Hibberd et al (1980) observed a similar effect of location on the nutritive characteristics of several grain sorghum hybrids obtained from different counties in Oklahoma. This is in agreement with the results in this study, although their results were not based on individual kafirins.

Of particular interest are the relationships among the kafirins and their relationships with feed quality characteristics. In this study, the correlations between CP and kafirin types were low, which agrees with results from previous studies (Sastry et al 1986). The significant negative relationships of  $\beta$ - $\alpha$ I with  $\gamma$  and  $\alpha$ II with  $\beta$ - $\alpha$ I observed in the current study highlight the complexity of the structure of kafirins. According to the literature,  $\alpha$ -kafirin constitutes 70–80% of total kafirin (Oria et al 2000). However, in this study, only  $\approx$ 18.15–25.46% of  $\alpha$ -kafirin migrated independently of other proteins. The bulk of it comigrated with  $\beta$ , forming  $\beta$ - $\alpha$ I. Under such circumstances, the significant high negative correlation between  $\alpha$ II and  $\beta$ - $\alpha$ I would be expected. The significant negative correlation between  $\beta$ - $\alpha$ I and  $\gamma$  and the lack of association between  $\alpha$ II and  $\gamma$  suggest some type of interaction between  $\beta$  and  $\gamma$  either at the level of synthesis or packaging into protein bodies. The low correlations of kafirin types with FAT and STA are understandable because these are biochemically different substances.

One of the most important components of this study involved characterizing the relationships of kafirin types with digestibility characteristics. IVDMD is an indirect measure of digestibility in the rumen. The sample is subject to microbial activity in the rumen fluid solution. However, whether microbial activity and rumen enzymes interact to break down the kafirins is not clear. In these experiments, no significant relationships occurred between IVDMD and kafirin types, indicating that IVDMD may not be a good measure of kafirin digestibility. This is in agreement with Pedersen et al (2000), who indicated that starch content has a greater impact on IVDMD measurements than do protein characteristics. The IVDMD in this study was conducted for 12 hr. Although most of the material is expected to pass the rumen at this time (Sniffen et al 1992; Pedersen et al 2000), whether digestion of the kafirin takes place in the rumen or elsewhere in the tract is not clear. The relationship between kafirin types and PD was of greater interest. Most reports in the literature indicate that the digestibility of grain sorghum is controlled by the structure of the protein body and the location of the kafirin proteins (Hamaker et al 1995; Oria et al 2000). However, significant correlations between  $\beta$ - $\alpha$ I and  $\gamma$  with

PD were noted in this study. This indicates that although kafirin packaging may be important in PD, kafirin type also plays a role. Previous research showed that kafirins are the last proteins to be digested (Hamaker et al 1986, Oria et al 2000), and that the addition of reducing agents improved their uncooked and cooked digestibilities (Hamaker et al 1987; Oria et al 1995b). Other studies also report that structural features within proteins, particularly disulfide bonds, negatively influence protein digestion (Oria et al 1995a). Differences in proportions of kafirin types may impact PD either through direct changes in chemical composition or resulting changes in protein structure. Some reports have indicated variation in digestibility among kafirin types (Oria et al 1995a). Evidence from a previous study indicates that poor digestibility of  $\alpha$ -kafirin may be related to its packaging near the interior of the protein bodies because disulfide bonds among  $\gamma$ - and  $\beta$ -kafirin form an enzyme-resistant structure that retards digestion of  $\alpha$ -kafirin at the protein body periphery (Oria et al 2000). Previous research also has indicated that the breakdown of kafirins starts on the outside of the protein bodies and progresses toward the interior (Rom et al 1992; Oria et al 2000).

The proportional variation of kafirin types indicates that parents with a good GCA for one kafirin would not necessarily be good for another kafirin. Also, the parents with good GCA for CP did not have good GCA values for all kafirin (Hicks et al unpublished data). Further studies should be conducted to understand the structure of proteins and especially kafirins, because they constitute the bulk of the total protein. One way to resolve this complex problem would be to identify and localize genes responsible for protein formation and for kafirin digestibility. Recently, Oria et al (2000) described a sorghum cultivar whose protein digestibility resembles or surpasses that of other cereals. This genotype may be useful for developing new lines to improve protein structure and digestibility of grain sorghum. Alternatively, this line could be used in identifying and localizing genes influencing digestibility, which subsequently could be used to enhance protein structure and digestibility of grain sorghum by means of marker-assisted selection or introgression.

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[Received October 23, 2000. Accepted April 4, 2001.]