Differences in Kafirin Composition During Endosperm Development and Germination in Sorghum Cultivars of Varying Hardness

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ABSTRACT

The content and composition of kafirins in hard- and soft-endosperm sorghum cultivars were studied during endosperm development and germination. Enzyme-linked immunosorbent assay, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, nonequilibrium pH gradient electrophoresis, and nitrogen analysis were used in the study. Kafirins extracted with 60% (v/v) *t*-butanol (Kafirin 1) increased slightly during endosperm development in both the cultivars. The predominant Kafirin 1 protein was α-kafirin. Kafirins extracted with 60% (v/v) *t*-butanol plus 5% (v/v) 2-mercaptoethanol (Karifin 2) increased more in both cultivars. The hard-endosperm kernels deposited the α- and γ-kafirins up to 14 days after anthesis (DAA). In the soft-endosperm kernels, these kafirins were deposited after 21 DAA. The β-kafirin was synthesized maximally after 21 DAA in both cultivars. An increased level of β-kafirin was noted in the hard-endosperm kernels at all stages of development. The β- and γ-kafirins became progressively cross-linked as endosperm development proceeded. Hard-endosperm kernels had more cross-linking than did soft-endosperm kernels. Qualitative differences within the individual kafirins during endosperm development were revealed by nonequilibrium pH gradient electrophoresis. A basic β-kafirin isoform was faintly stained in extracts of soft-endosperm kernels. During germination, the β- and γ-kafirins were extensively degraded in both cultivars. More kafirin degradation occured in the soft-endosperm kernels than in the hard-endosperm kernels. Nonequilibrium pH gradient electrophoresis indicated a differential breakdown of the individual kafirins during germination. Differences in the rate, type, and content of kafirin deposition affected protein degradation during germination.

Sorghum (*Sorghum bicolor* (L.) Moench) is an important crop of the semiarid tropics. One of the most important varietal differences is the proportion of outer, hard, translucent endosperm to inner, soft, opaque endosperm. This difference is used to classify sorghums as hard or soft. Hard-endosperm kernels have superior agronomic characteristics and contain more prolamin proteins than do soft-endosperm kernels (Chandrashekar and Kirleis 1988). Attempts at increasing the lysine content in sorghum and maize resulted in grains with soft endosperm (Singh and Axtell 1973, Ortega and Bates 1983). The amount of alcohol-soluble protein in sorghum and maize is lower in high-lysine grains than in normal grains (Gentinetta et al 1975, Jambunathan et al 1975, Paulis and Wall 1979). An increased level of γ-zein was recently demonstrated in several hard maize genotypes that are high in lysine (Wallace et al 1990, Paiva et al 1991, Paulis et al 1991). Such differences in the prolamin composition of normal, hard-, and soft-endosperm kernels have not been studied in sorghum.

The seed prolamin content increases almost linearly between the milk stage and the hard-dough stage in developing sorghum kernels (Taylor et al 1985a). Hard-endosperm kernels develop faster and have a higher concentration of protein bodies in the outer endosperm than do the softer endosperm kernels (Shull et al 1990).

The prolamins in sorghum show the greatest decrease during germination (Taylor 1983). The protein bodies were progressively hydrolyzed from their outer surface (Taylor et al 1985b). Endosperm modification begins at the scutellum interface and subsequently moves into the soft endosperm; the hard endosperm is modified last (Glennie et al 1983). Mohammad and Esen (1990) studied the pattern and mode of degradation of zein components during germination and postulated the arrangement of the different zein polypeptides within the protein body.

The purpose of this study was to investigate the changes in composition of kafirins during endosperm development in normal, hard-, and soft-endosperm sorghum cultivars. A further objective was to determine the relationship of the developmental profile of prolamins and their hydrolysis during germination, since kafirins are storage proteins deposited during kernel development to be used up during germination. A greater understanding of sorghum grain hardness and the role of prolamins in germination will thus be acquired.

MATERIALS AND METHODS

Sample Preparation

Two low-tannin, nonpigmented sorghum cultivars, E-35-1 (hard) and SPV-104 (soft), were obtained from the International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India. Grain hardness was determined using the Kiya hardness tester (Kiya Seisakusho Ltd., Tokyo). The hard and soft cultivars showed a hardness value of 13.4 and 4.0 kg/cm^2, respectively. The relative proportions, by weight, of corneous and floury endosperm were 78.9 and 21.1% in E-35-1 and 66 and 34% in SPV-104, respectively.

The two cultivars were planted in plots in the October-December 1991 season. The individual plants were identified and tagged immediately after anthesis; grains were collected at 14, 21, 28, and 42 days after anthesis (DAA). The panicles were frozen at −50°C. The grains from the middle part of the panicle were harvested, and the pericarp and germ of these kernels were manually removed. The endosperm was then freeze-dried, and the dry weight of 30 endosperms was determined. The endosperms were ground into flour using a mortar and pestle and passed through a 0.4-mm sieve. The overs were reground and passed through the same sieve. The grains collected at 42 DAA were decorticated in a Satake grain testing machine (Satake Engineering, Tokyo), ground into flour in a Udy cyclone mill (Udy Corp, Fort Collins, CO), and passed through a 0.4-mm sieve.

For germination, 100 g of mature grain was steeped overnight in 500 ml of distilled water and then germinated between blotting papers at 25°C. Samples were drawn at 0–6 days after germination (DAG). The roots, shoots, germ, and pericarp were manually removed. The grains were then freeze-dried. The dry weight of 30 endosperms was determined. The endosperms were ground into flour in a mortar and pestle and passed through a 0.4-mm sieve.

Kafirin Extraction

For nitrogen analysis, 500 mg of flour was extracted either with 10 ml of 60% (v/v) *t*-butanol alone or with 60% (v/v) *t*-butanol plus 5% (v/v) 2-mercaptoethanol. These extracts are designated as Kafirin 1 and Kafirin 2, respectively. After three extractions, the supernatants were combined and dried at 80°C. Extractions were carried out in duplicate. Nitrogen was determined by the Kjeldahl method (AOAC 1975). Protein was

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calculated using a factor of 6.25. For enzyme-linked immunosorbent assay (ELISA), 50 mg of flour was extracted with 0.4 ml of solvents in Eppendorf tubes, vortexed for 5 min, and centrifuged at 4,000 × *g* for 20 min. The extract was diluted 1:5,000 in 60% (v/v) *t*-butanol containing 0.6% (v/v) 2-mercaptoethanol. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), samples were prepared in the same manner and mixed with an equal volume of SDS sample buffer containing 1.23*M* Tris, 10% (w/v) SDS, and 5% (v/v) 2-mercaptoethanol. The samples were boiled for 3 min and cooled before use. For nonequilibrium pH gradient electrophoresis (NEPHGE), the kafirin extracts were mixed with an equal volume of lysis buffer containing 4*M* urea, 2% (w/v) NP-40, and 1% (w/v) dithiothereitol and used directly.

Preparation of Antibodies

Polyclonal antibodies against the purified α-, β-, and γ-kafirins were raised in rabbits as described by Mazhar et al 1993.

ELISA

Indirect ELISA was carried out on 96-well Immulon microtiter plates as reported previously (Mazhar et al 1993). All assays were performed in triplicate. Kafirins are very hydrophobic proteins, so the pure freeze-dried samples are difficult to solubilize in any solvent that is suitable for ELISA. Standard curves were not prepared for them. Instead, all values were normalized against a mature hard cultivar (Wallace et al 1990).

TABLE I
Content and Composition of Kafirin 1 (extracted with 60%, v/v, *t*-butanol) During Endosperm Development in Sorghum Cultivars of Varying Hardness[a]

Cultivar	DAA[b]	Kafirin-1[c]	α-Kafirin[d]	β-Kafirin[d]	γ-Kafirin[d]
E-35-1	14	0.383 a	26.6 a	8.1 a	6.3 a
(Hard)	21	0.638 b	41.4 b	23.2 b	9.3 b
	28	0.710 c	46.4 c	29.3 c	15.3 c
	42	0.816 d	34.4 d	19.0 d	13.5 d
SPV-104	14	0.258 a	12.9 a	15.0 a	3.6 a
(Soft)	21	0.442 b	23.2 b	16.8 b	8.2 b
	28	0.490 c	26.2 c	13.8 a	9.7 c
	42	0.561 d	27.3 c	10.7 c	9.7 c

[a]Means followed by different letters within a column for each variety are statistically different at $P < 0.05$ level.
[b]Days after anthesis.
[c]Mean dry weights per endosperm (mg) at 14, 21, 28, and 42 DAA are 9.6, 15.9, 23.6, and 27.7 for E-35-1 and 7.8, 14.6, 20.4, and 23.3 for SPV-104, respectively. Values are milligrams per endosperm (N × 6.25).
[d]Relative amounts of α-, β-, and γ-kafirins estimated by enzyme-linked immunosorbent assay. All values normalized to those of E-35-1 (42 DAA) sample in Table II.

TABLE II
Content and Composition of Kafirin 2 (extracted with 60%, v/v, *t*-butanol plus 5%, v/v, 2-mercaptoethanol) During Endosperm Development in Sorghum Cultivars of Varying Hardness[a]

Cultivar	DAA[b]	Kafirin-2[c]	α-Kafirin[d]	β-Kafirin[d]	γ-Kafirin[d]
E-35-1	14	0.447 a	24.4 a	19.1 a	28.2 a
(Hard)	21	0.852 b	38.3 b	43.4 b	38.7 b
	28	1.314 c	67.4 c	66.8 c	74.2 c
	42	1.796 d	100.0 d	100.0 d	100.0 d
SPV-104	14	0.245 a	16.1 a	9.9 a	14.5 a
(Soft)	21	0.481 b	38.7 b	35.1 b	39.1 b
	28	0.712 c	66.7 c	43.2 c	61.6 c
	42	1.092 d	83.1 d	67.3 d	70.4 d

[a]Means followed by different letters within a column for each variety are statistically different at $P < 0.05$ level.
[b]Days after anthesis.
[c]Mean dry weights per endosperm (mg) at 14, 21, 28, and 42 DAA are 9.6, 15.9, 23.6, and 27.7 for E-35-1 and 7.8, 14.6, 20.4, and 23.3 for SPV-104, respectively. Values are milligrams per endosperm (N × 6.25).
[d]Relative amounts of α-, β-, and γ-kafirins estimated by enzyme-linked immunosorbent assay. All values have been normalized to those of E-35-1 (42 DAA) sample.

SDS-PAGE

SDS-PAGE was conducted at a constant current of 50 mA on a horizontal Multiphor II electrophoresis system (LKB, Bromma, Sweden) according to the procedure of Laemmli (1970). Electrophoresis was carried on 15% (w/v) acrylamide gels. Standard proteins (phosphorylase b [97,400], glutamate dehydrogenase [55,400], lactate dehydrogenase [36,500], and trypsin inhibitor [20,100]) were obtained from Boehringer Mannheim.

NEPHGE

Nonequilibrium pH gradient electrophoresis was conducted on a horizontal Multiphor II electrophoresis system according to Bravo (1984). Electrofocusing was carried out on 4% (w/v) acrylamide gels containing 6*M* urea, 2% NP-40, 3.4% pH 5–8 Pharmalyte, and 0.85% each of pH 8–10.5 and pH 2.5–5 Pharmalyte. Gels were run at 400 V for 3 hr and stained with 0.05% (w/v) Coomassie Brilliant Blue R-250 in a mixture of trichloro acetic acid, acetic acid, and methanol (10:10:25). Gels were destained in the same solution without the dye.

Statistical Analysis

All estimations were made at least in duplicate. Data was subjected to a one-way analysis of variance. The level of significant difference was measured at the 0.05% level using the KWIKSTAT computer program.

RESULTS

Kafirin Content During Development

Contents of the Kafirin 1 at various stages of endosperm development are shown in Table I. The amount of Kafirin 1 in E-35-1 increased by 113% from 14 to 42 DAA; in SPV-104, the amount increased by 117%. The relative amounts of α-, β-, and γ-kafirins were estimated by an indirect ELISA procedure. At all stages of development, the α-kafirins were predominant. In E-35-1, the α-kafirins increased 74% from 14 to 28 DAA. They decreased 25% thereafter. However, the β- and γ-kafirins increased 260 and 142%, respectively, (14–28 DAA), decreasing 35 and 11% thereafter. In SPV-104, the α- and γ-kafirins increased 103 and 170%, respectively, (14–28 DAA), remaining steady thereafter. The β-kafirin increased 12% (14–21 DAA), decreasing 36% (21–42 DAA).

Contents of Kafirin 2 increased steadily during endosperm development in both cultivars (Table II). In E-35-1, Kafirin 2 increased 300% (14–42 DAA); in SPV-104, it increased 344%. ELISA results revealed that, in E-35-1, the α- and γ-kafirins were deposited by 14 DAA. In SPV-104, these kafirins were deposited mostly after 21 DAA. The β-kafirin, however, was deposited maximally after 21 DAA in both cultivars. The α-kafirin

TABLE III
Content and Composition of Kafirin 2 (extracted with 60%, v/v, *t*-butanol plus 5%, v/v, 2-mercaptoethanol) During Germination in Sorghum Cultivars of Varying Hardness[a]

Cultivar	DAG[b]	Kafirin-2[c]	α-Kafirin[d]	β-Kafirin[d]	γ-Kafirin[d]
E-35-1	0	1.796 a	100.0 a	100.0 a	100.0 a
(Hard)	2	1.568 b	82.1 b	76.7 b	67.0 b
	4	1.328 c	74.3 c	53.4 c	44.2 c
	6	1.104 d	57.2 d	37.5 d	43.9 c
SPV-104	0	1.092 a	83.1 a	67.3 a	70.4 a
(Soft)	2	0.752 b	53.5 b	48.9 b	66.2 b
	4	0.430 c	41.7 c	24.1 c	32.2 c
	6	0.324 d	18.9 d	7.6 d	8.1 d

[a]Means followed by different letters within a column for each variety are statistically significant at $P < 0.05$ level.
[b]Days after germination.
[c]Mean dry weights per endosperm (mg) at 0, 2, 4, and 6 DAG are 27.7, 27.0, 25.9, and 22.1 for E-35-1 and 23.3, 22.8, 19.4, and 18.0 for SPV-104, respectively. Values are milligrams per endosperm (N × 6.25).
[d]Relative amounts of α-, β-, and γ-kafirins were estimated by enzyme-linked immunosorbent assay. All values have been normalized to those of E-35-1 (0 DAG) sample.

increased 300% in E-35-1 and 416% in SPV-104 (14–42 DAA). The β- and γ-kafirins increased 420 and 250% in E-35-1 (14–42 DAA) and 570 and 380% in SPV-104 (14–42 DAA). Greater deposition of all the three kafirins at 14–42 DAA occurred in the soft-endosperm kernels. The amount of α-kafirin at each developmental stage was similar for both soft- and hard-endosperm cultivars. The amount of β-kafirin, however, was higher in the hard-endosperm cultivar at all stages of development. The level of kafirin was also higher in the hard-endosperm kernels at all developmental stages, except at 21 DAA.

Kafirin Content During Germination

Upon germination, the Kafirin 2 content decreased 38% in E-35-1 and 70% in SPV-104 (0–6 DAG) (Table III). The α-kafirins fell 43% in E-35-1 and 77% in SPV-104 (0–6 DAG). The β- and γ-kafirins were degraded at a faster rate than the α-kafirins in both cultivars. In E-35-1, the β- and γ-kafirins were reduced by 63 and 56%, respectively (0–6 DAG). In SPV-104, they were reduced by 89 and 88%, respectively.

SDS-PAGE and NEPHGE

Kafirins subjected to SDS-PAGE separate into three distinct bands: α_1-kafirin (28–24 kDa), α_2-kafirin (22 kDa), and β-kafirin (19 kDa). In addition, two bands of 14 and 10 kDa are also seen, along with a 48-kDa band that is a dimer of the 24-kDa kafirin. According to previous reports and work carried out in our laboratory, a water-soluble 27-kDa protein (γ-kafirin) is extracted from total kafirin (Evans et al 1987; Taylor et al 1989; Watterson et al 1990; Mazhar et al 1993). The SDS-PAGE system was unable to separate the α_1- and γ-kafirins because of the overlapping of the two proteins.

The SDS-PAGE profiles of Kafirin 1 and Kafirin 2 extracts from the endosperm of two cultivars at various developmental stages are shown in Figure 1. All the Kafirin 1 bands were faintly stained during endosperm development in both the cultivars (Lanes 1–8). The intensities of the Kafirin 2 bands, however, increased during endosperm development (Lanes 9–16). The β-kafirin band (19 kDa) was faintly stained at 14 DAA and increased in intensity thereafter in both cultivars (Lanes 9, 13).

Kafirins were separated into 10 bands on NEPHGE (Fig. 2). The bands were numbered 1–10 from the acidic to the basic end of the gel. The bands belonging to the three different kafirins were identified by Western blotting (*data not shown*). The α-kafirin antibody reacted with five bands (1, 5, 6, 7, 9). The β-kafirin antibody reacted with three bands (3, 4, 10). The γ-kafirin antibody reacted with only one band (8). Band 2 did not react with any of the three antibodies used.

The NEPHGE profiles of Kafirin 1 and Kafirin 2 extracts from the endosperm of two cultivars at various stages of development are shown in Figure 2. In Kafirin 1, one of the α-kafirin bands (5) was the most prominent band in both cultivars. The intensity of the other α-kafirin bands (6, 7) decreased during development. The γ-kafirin band (8) was faint in the soft-endosperm cultivar at maturity (Lane 8). In Kafirin 2, one of the α-kafirin bands (1) was visible in the hard-endosperm cultivar and not in the soft; the other α-bands were also more intense in the hard-endosperm kernels. One of the basic β-kafirin isoforms (10) was faintly stained in the soft-endosperm kernels at all stages of endosperm development. The single γ-kafirin band (8) was particularly faint in the soft-endosperm kernels at maturity (Lane 16).

The SDS-PAGE profiles of Kafirin 2 extracts from germinating sorghum are shown in Figure 3. The kafirin profile of E-35-1 did not change, except that the staining intensity of all the bands decreased at 4 DAG (Lanes 1–7). In SPV-104, the staining intensity of the bands decreased at 3 DAG. The β-kafirin band was not detected at 4 DAG. At 6 DAG, only the α-kafirin band was visible (Lanes 8–14).

The NEPHGE profiles of Kafirin 2 extracts from germinating sorghum are shown in Figure 4. The Kafirin 2 profiles in the hard-endosperm kernels were similar, except that the γ-kafirin band (8) became fainter at 2 DAG (Lanes 1–7). In the soft-endosperm kernels, one of the α-kafirin isoforms (1) was degraded at 3 DAG. The acidic β-kafirin isoforms (3, 4) were faintly stained at 3 DAG. The basic β-kafirin isoform (10) was completely digested at 4 DAG. At 6 DAG, only the α-kafirin isoforms (5, 6, 7, 9) were prominently visible (Lanes 8–14).

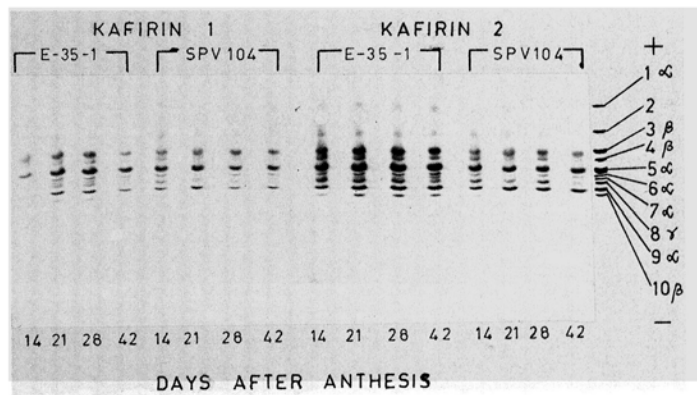

Fig. 2. Nonequilibrium pH gradient electrophoresis profile of kafirins during sorghum endosperm development 14, 21, 28, and 42 days after anthesis. Kafirin 1 extracted with 60% (v/v) *t*-butanol; Kafirin 2 extracted with 60% (v/v) *t*-butanol plus 5% (v/v) 2-mercaptoethanol. Cultivars E-35-1 and SPV-104. Numbers in right margin indicate bands appearing from acidic to basic end of the gel. The suffix indicates reaction with different antibodies.

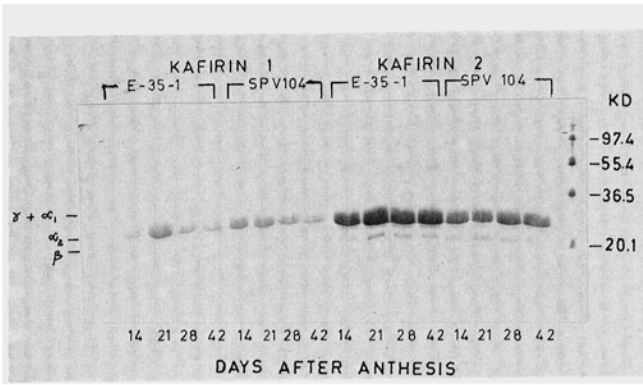

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of kafirins during sorghum endosperm development 14, 21, 28, and 42 days after anthesis. Kafirin 1 extracted with 60% (v/v) *t*-butanol; Kafirin 2 extracted with 60% (v/v) *t*-butanol plus 5% (v/v) 2-mercaptoethanol. Cultivars E-35-1 and SPV-104. Low molecular weight protein standard in extreme right lane.

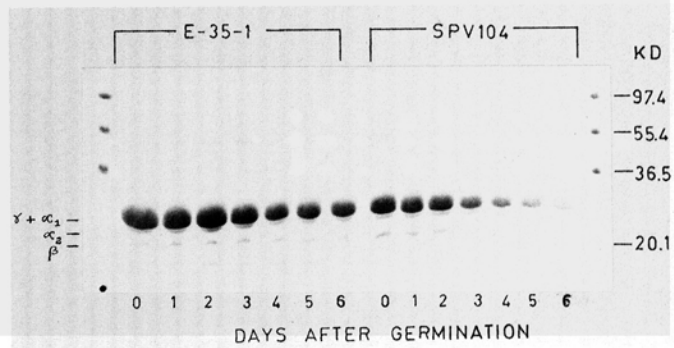

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of kafirins during sorghum endosperm 0–6 days after germination. Kafirin 2 extracted with 60% (v/v) *t*-butanol plus 5% (v/v) 2-mercaptoethanol. Cultivars E-35-1 and SPV-104. Low molecular weight protein standards in extreme left and right lanes.

DISCUSSION

Initially, kafirins extracted with aqueous alcohol increased during kernel development in both opaque and normal sorghums; thereafter they decreased in the opaque cultivar. Kafirins extracted with aqueous alcohol and reducing agent increased continuously throughout endosperm development in both opaque and normal sorghums (Van Scoyoc et al 1988). The content of Kafirin 1 and Kafirin 2 increased in both hard- and soft-endosperm kernels, but the Kafirin 2 increased at a faster rate. This may be due to the increased extractability of β- and γ-kafirins, which become progressively cross-linked as endosperm development proceeds.

In maize, a decrease in the α-zein was followed by a two- to threefold increase in the γ-zein in several hard-endosperm maize genotypes (Wallace et al 1990). Kodrzycki et al (1989) reported a decrease in the transcription of α-zein gene during endosperm development in *opaque*-2 maize. In our study, the α-kafirin component in the Kafirin 2 extract increased similarly in both cultivars as development proceeded. The level of β-kafirin, however, was higher in the hard-endosperm kernels at all stages of development. The hard-endosperm kernels deposited twice the amount of α- and γ-kafirins at 14 DAA. A comparison of the composition of Kafirin 1 and Kafirin 2 revealed that the β- and γ-kafirins became progressively more cross-linked in the hard-endosperm kernels. It may be postulated that the formation of vitreous structures through a disulfide-bonded network involves β- and γ-kafirins because the proportion of the corneous endosperm is far greater in the hard-endosperm kernels at maturity.

Taylor (1983) reported a decrease in prolamin content from 45 to 16% in sorghum germinated over a period of seven days. According to Wu and Wall (1980), the cross-linked kafirin decreases more than the noncross-linked kafirin during germination. A major kafirin protein (26 kDa) decreased in protein body preparations from sorghum germinated over a period of seven days (Taylor et al 1985a). According to Mohammad and Esen (1990), γ-zeins are the first zeins to be degraded, followed by the β-zeins, in maize during germination. The α-zeins were the most resistant; a small amount was visible on immunoblots even at 26 DAG. These results were also supported by de Barros and Larkins (1990).

In sorghum, grain hardness affected the rate of degradation of the kafirins. The β- and γ-kafirins were the most susceptible kafirins. The β- and γ-kafirins are located on the periphery of the protein bodies (Shull 1991). A similar location of the γ-zein has been reported in maize (Lending and Larkins 1989). Because protein body degradation occurs through surface pitting during germination (Taylor et al 1985b), maximal breakdown of the β- and γ-kafirins may be explained by their peripheral location on the protein bodies. Further, Glennie et al (1983) suggested that proteins in sorghum are modified from the floury to the corneous endosperm during germination. Differential concentration of the various kafirins in different parts of the endosperm also could result in their differential degradation during germination. The β-kafirins are more abundant and more cross-linked in the hard-endosperm kernels, so the β-kafirin could protect the α-kafirin from digestion during germination. The β-kafirins are less abundant and less cross-linked in the soft-endosperm kernels, so more rapid degradation of α-kafirin is expected.

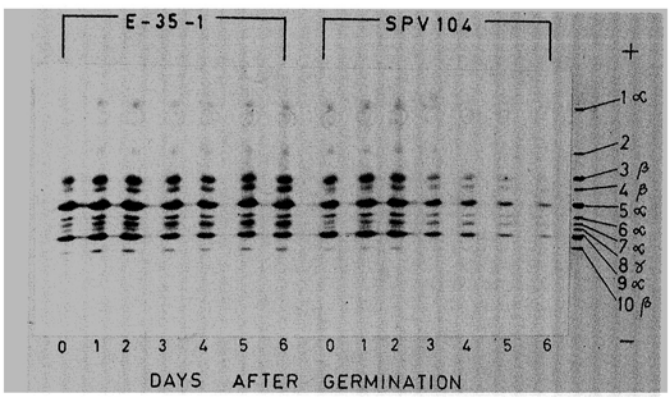

Fig. 4. Nonequilibrium pH gradient electrophoresis profile of kafirins during sorghum endosperm 0–6 days after germination. Kafirin 2 extracted with 60% (v/v) *t*-butanol plus 5% (v/v) 2-mercaptoethanol. Cultivars E-35-1 and SPV-104. Numbers in right margin indicate the bands appearing from acidic to basic end of the gel. The suffix indicates reaction with different antibodies.

CONCLUSION

Developmental differences were observed in the rate of deposition of the three kafirins. The hard-endosperm kernels deposited more β-kafirins than did the soft-endosperm kernels during all stages of development. The extent of cross-linking of the β- and γ-kafirins was also greater in the hard-endosperm kernels. Hence, in sorghum, grain hardness may be a function of kafirin composition. Changes in kafirin composition during endosperm development corresponded to changes in the rate of degradation during germination. Kafirins in the hard-endosperm kernels were less degraded during germination. The NEPHGE and ELISA analyses effectively revealed differences in kafirins during endosperm development and germination. They could be used for varietal selection and screening during malting.

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