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Genetic Variability of Alcohol-Soluble Storage Proteins in High-Lysine Sorghums

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The genetic variability of the alcohol-soluble storage proteins in normal- and high-lysine sorghum grains was examined. The yields of kafirins and alcohol-soluble reduced glutelins (ASG) and their isoelectric focusing (IEF) patterns and reversed-phase high-performance liquid chromatography (HPLC) elution chromatograms were determined on the normal hybrid TE77 and inbred P721 and on the high-lysine line IS-11758 and the chemically induced high-lysine opaque mutant P721*O*. The results confirmed that kafirin and ASG contents of high lysine lines were reduced and that the mutant gene (*O*) did not alter the composition of the alcohol-soluble proteins in P721. Studies were also conducted on inbreds derived from crosses of P721*O* and high-yielding elite sorghum inbreds (954063, IS-4404). The IEF and HPLC patterns of kafirins and ASG proteins of these inbreds compared to those of the parent lines suggested that the genes coding kafirins and ASG proteins were located on at least two different chromosomes and one of those chromosomes may also contain the *O* gene.

A major problem in food use of sorghum grain is the poor nutritional quality of its protein (Wall and Paulis, 1978). Three factors contribute to the low quality of sorghum protein: (1) low solubility in aqueous media; (2) insolubilization of proteins by tannins present in grain pericarp and testa (Butler et al., 1984); (3) deficiencies in essential amino acids, especially lysine. Singh and Axtell (1973) discovered two lines of Ethiopian sorghums that had higher lysine contents in the grain protein. Grains of these lines, which contain the mutant recessive gene *hl*, had floury shrunken kernels and high tannin contents. Efforts to improve yields and kernel characteristics were not successful. Later, Mohan and Axtell (1975) found among chemically induced mutants of the inbred P721 a floury high-lysine line, P721 *Opaque* (*O*). This mutant strain had 40% more lysine in its protein but also had soft opaque dented kernels and gave lower yields. Purdue University researchers (Van Scoyoc, 1979) have crossed these high-lysine lines with elite inbreds, used to produce high-yielding hybrids, and selected improved progeny lines having better yields with improved grain protein quality.

The high-lysine sorghum grain endosperms are characterized by reduced levels of the prolamin, kafirin, and the alcohol-soluble reduced glutelins (ASG) (Jambunathan et al., 1975; Guiragossian et al., 1978; Paulis and Wall, 1979). Glutelins are those proteins that are insoluble in saline or aqueous alcohol solutions but are soluble in dilute alkali. They may also be dissolved in detergent or urea solutions after disruption of the disulfide bonds that link their polypeptide chains. The alcohol-soluble glutelin (ASG) is that fraction of the glutelin that may be dissolved in 60% *tert*-butyl alcohol in the presence of a reducing agent such as 2-mercaptoethanol (ME). Kafirins and ASG are deficient in lysine and are major fractions of the total protein of sorghum endosperm (Guiragossian et al., 1978; Paulis and Wall, 1979). These proteins are mixtures of closely related proteins that can be resolved into many components by electrophoresis with acidic buffers in polyacrylamide gels (Paulis and Wall, 1979). Paulis and Wall also demonstrated by polyacrylamide electrophoresis in sodium dodecyl sulfate containing buffers that both kafirins and ASG consisted of several different species exhibiting varied molecular weights, the main ones being 21 000 and 23 000 Da. Recently, Sastry et al. (1986) have shown that the kafirin and ASG polypeptides of sorghum can also be separated by isoelectric focusing (IEF) in polyacrylamide gels or by reversed-phase (RP) high-performance liquid chromatography (HPLC) to yield separation patterns that are characteristic of the different sorghum varieties from which the proteins were extracted. The sorghum kafirins and ASG polypeptides were shown to be highly homologous and coded by a multigene family. Allelic genes were codominant as deduced by comparison of the IEF and the HPLC separation patterns of kafirins and ASG polypeptides extracted from grains of inbreds and those from hybrids resulting from their crosses. Proteins from both parents were present in extracts of hybrids, but individual alcohol-soluble proteins inherited from the female parent were generally in greater concentration than proteins inherited from the male because endosperm is triploid with two sets of chromosomes derived from the female.

Unlike the recessive mutant genes *opaque 2* or *fluory 2* of corn or the *hl* gene in the Ethiopian sorghum, IS-11758, the P721*O* gene that elevates endosperm lysine content is partially dominant. In the present work we have sought to establish whether this gene selectively curtails production of certain kafirin or ASG proteins. We were interested in determining whether grain protein lysine level was inversely related to the proportion of kafirin plus ASG of the total protein. Also the question of whether the *O* gene is linked on the same chromosome with some of the genes coding for kafirins and ASGs was investigated. For these purposes we separated and determined by IEF and HPLC kafirins and ASG polypeptides in extracts from grains of normal- and high-lysine sorghums. Special emphasis was placed on analysis of extracts of P721, P721*O*, and inbred lines derived from F_2 generations of crosses of P721*O* with elite high-yielding vitreous sorghum lines. Additional knowledge of the mechanism of action of the *O* gene may facilitate breeding of better yielding high-lysine sorghums.

MATERIALS AND METHODS

Sorghum Grains. Normal vitreous sorghum grains investigated in this study included the hybrid Taylor-Evans 77 (TE77), inbreds P721 and 954063, and the pig-

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Table I. Protein Yields from Normal- and High-Lysine Sorghums

variety	protein,[a] %	lysine,[a] % of protein	% of grain protein[b] kafirin K	ASG	K + ASG
IS11758	15.0 ± 0.2	3.13 ± 0.1	14.5 ± 1.4	24.2 ± 3.2	42.1 ± 2.0
TE77	10.7 ± 0.3	1.80 ± 0.0	27.2 ± 2.1	25.7 ± 5.2	57.8 ± 3.1
P721	13.1 ± 0.1	2.0 ± 0.1	29.8 ± 1.5	24.3 ± 1.5	58.5 ± 4.0
P721*O*	15.4 ± 0.2	2.95 ± 0.2	19.5 ± 3.7	17.7 ± 3.3	41.9 ± 0.4
964063	11.1 ± 0.1	2.24 ± 0.1	30.8 ± 1.8	31.8 ± 2.2	66.3 ± 2.1
opaque derived inbreds (P721*O* × 964063)					
entry 94	11.3 ± 0.1	3.1 ± 0.2	22.4 ± 1.1	21.0 ± 2.2	46.8 ± 2.5
entry 120	10.8 ± 0.1	3.0 ± 0.1	20.5 ± 1.0	19.8 ± 1.8	47.3 ± 0.5
IS-4404[c]	10.9 ± 0.3	2.2 ± 0.0	31.82 ± 0.3	25.3 ± 7.1	61.0 ± 3.0
opaque derived inbreds (P721*O* × 4404)					
entry 6	12.6 ± 0.4	3.3 ± 0.0	20.9 ± 1.4	22.2 ± 2.4	42.7 ± 0.8
entry 18	12.4 ± 0.3	3.3 ± 0.1	17.2 ± 1.4	18.9 ± 3.5	40.8 ± 1.1
entry 81	11.8 ± 0.2	3.2 ± 0.2	23.2 ± 0.5	19.0 ± 3.7	46.1 ± 0.2
entry 149	13.0 ± 0.1	3.2 ± 0.1	18.1 ± 0.1	16.8 ± 3.0	40.8 ± 0.4

[a] Results of duplicate analyses. [b] Results of duplicate extractions. [c] Dehulled.

mented line IS-4404. High-lysine sorghums included the Ethiopian cultivar IS-11758 with recessive mutation, *hl*, and the chemically induced mutant P721*O*. Also, two opaque F_7 generation inbreds selected from the cross between P721*O* and 954063 (entries 94 and 120) and four opaque F_7 inbreds selected from the cross P721*O* and line IS-4404 (entries 6, 18, 81, and 149) were investigated. All grain was grown at West Lafayette in 1978 under conditions detailed by Van Scoyoc (1979). The initial derived *opaque* inbred lines were selfed through six generations to ensure homozygous status. The yields of entries 120 and 81 were equal or better than their elite vitreous parents (Van Scoyoc, 1979). The line IS-4404 was highly pigmented due to anthocyanins in testa and pericarp. A sample was dehulled with a Strong-Scott laboratory model barley pearler. Protein extracts were prepared from both the whole-grain and dehulled samples, and their compositions were compared.

Protein Extraction. The whole grain or dehulled grain was ground in a Udy laboratory cyclone mill to −40 mesh. The ground meal was defatted by stirring with 10 volumes of hexane at 4 °C for 1 h and filtered under vacuum on a Buchner funnel. The kafirins were extracted from 3.5-g portions of the defatted meals in 60-mL polypropylene centrifuge tubes with 35 mL of 60% *tert*-butyl alcohol by vigorously shaking on a reciprocal shaker for 2 h at room temperature. The suspension was centrifuged in a Model CM International centrifuge at 3000 rpm for 20 min. After the supernatant was decanted, the residual meal was reextracted with 17.5 mL of 60% *tert*-butyl alcohol for 1 h. After centrifugation, the kafirin extracts were combined. To extract ASG, the residual meal was extracted first with 35 mL of 60% *tert*-butyl alcohol containing 2% 2-mercaptoethanol (ME) for 2 h at room temperature and then with 17.5 mL of that solvent for 1 h as described above. Other 3.5-g defatted samples of the meals were extracted directly with 35 mL of 60% *tert*-butyl alcohol containing 2% ME for 2 h and then with 17.5 mL of that solvent for 1 h to remove total kafirin plus ASG (K + ASG). These latter extracts were prepared because earlier experience indicated that better yields of total K + ASG were obtained in this manner and we were interested in relating the ratio of K + ASG to total protein to the lysine levels in the grain. All extracts were prepared in duplicate, analyzed for nitrogen, dialyzed, and freeze-dried.

Reduction with ME and alkylation with acrylonitrile of 2 mg of protein in 100 μL of 8 M urea was conducted in 1.5-mL polypropylene centrifuge tubes as described by Paulis and Wall (1977). After acidification with aluminum lactate buffer, the samples were centrifuged 20 min in an Eppendorf centrifuge. In some experiments the kafirins were not reduced prior to IEF or HPLC in order to compare separations of native proteins to those that were reduced and alkylated. For HPLC 50 μL of supernatant reduced–alkylated protein solution was diluted with 5 volumes of 6 M guanidine hydrochloride and centrifuged further in a Beckman TL 100 centrifuge at 80000 rpm.

Analysis of Meals and Extracts. Protein contents of the flour and various extracts were determined by multiplying their nitrogen contents as determined by semimicro Kjeldahl analysis by 6.25. Lysine values of the whole grain were determined with a Beckman 120C amino acid analyzer after hydrolysis with 6 N HCl (Van Scoyoc, 1979).

Isoelectric Focusing and HPLC of Protein Extracts. Isoelectric focusing of proteins in thin polyacrylamide gels [5% acrylamide and 0.183% *N,N'*-methylenebis(acrylamide))] containing 8 M urea and 2% pH 6–8 ampholines was carried out on an LKB Multiphor horizontal electrophoresis apparatus as described by Wall et al. (1984). The separation of reduced–alkylated proteins from solutions absorbed on filter paper wicks was conducted at 4 °C with 13-W constant power for 4 h. The proteins on the gels were visualized by the silver stain reagent kit of the BioRad Corp. (Richmond, CA) following the directions of the manufacturer.

Separation of the alcohol-soluble proteins by RP-HPLC was conducted on a Waters gradient HPLC system consisting of M6000A and M45 pumps controlled by a Model 660 solvent programmer and a WISP 710B autoinjector. The proteins (100 μL) were separated at 30 °C on a Synchropak RP-P (C_{18}) 300-Å pore size column (SynChrom Inc., Linden, IN), 250 × 4.0 mm, with a flow rate of 1 mL/min using a linear gradient of 47.5–57.25% acetonitrile in water containing 0.1% trifluoroacetic acid over 60 min and 10 min at the final solvent concentration. The eluent was then restored to the initial composition by a linear gradient over 10 min, and the column was equilibrated for 10 min more with that solution. Proteins were determined in the effluent by monitoring at 210 nm with a Waters Model 450 variable-wavelength detector.

RESULTS

Protein Extraction Yields. In Table I are summarized protein analyses and lysine contents of the proteins in grains from the cultivars investigated. Also the yields of kafirins, ASG, and K + ASG extracted from these grains are given. Some differences in duplicate extractions of kafirins or ASG from the same meal occurred. Possibly some kafirins were disulfide linked and extracted with ASG. TE77, a standard U.S. hybrid, IS-11758, an Ethiopian high-lysine grain, and P721, an inbred line from which the P721*O* high-lysine mutant was derived, were used as

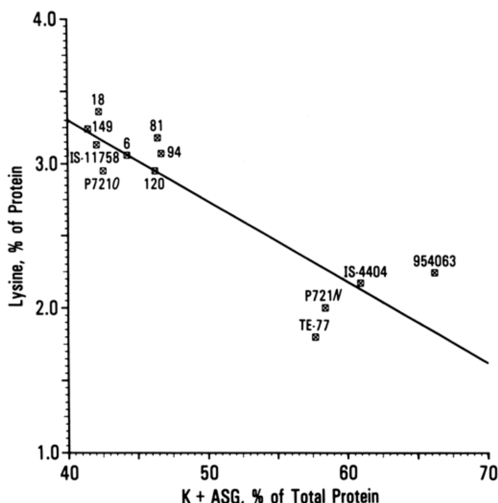

Figure 1. Lysine content of protein of normal- and high-lysine sorghums vs. percent of protein of kafirin plus alcohol-soluble glutelin (K + ASG). Linear regression equation is % lysine in protein = 5.545 − 0.0560 % K + ASG of grain protein; standard deviation = 0.257; correlation coefficient (*r*) is −0.89.

reference materials since they were investigated earlier in this laboratory (Paulis and Wall, 1979). In the earlier study the salt-soluble albumins and globulins were extracted prior to extraction of alcohol-soluble proteins. However, the yields of kafirins and ASGs from P721 and TE77 were similar in the present work (Table I) to that obtained previously. Combined yields of separately extracted kafirin and ASG were similar for the two extraction methods from the high-lysine grains P721*O* and IS-11758. Yields of kafirins in the various cultivars varied from 14 to 32%, of ASG from 18 to 32%, and of K + ASG from 41 to 66%. The yield of K + ASG protein consistently exceeded the sum of these two fractions when they were extracted separately. This difference in extraction yields may be caused by protein denaturation during the longer extraction entailed by the two-step procedure. The yields of K, ASG, and K + ASG were higher for the normal grains TE77 and P721 than for the high-lysine grains IS-11758 and P721*O*, as observed earlier.

The elite vitreous inbred 964063 had high contents of kafirin and ASG (Table I). In contrast, lesser amounts of kafirin and ASG were extracted from the tannin-rich normal IS-4404 before dehulling, but the dehulled sample yielded amounts of these proteins similar to other normal grains (Table I). The opaque higher lysine lines (entries 94 and 120) derived from the crosses of P721*O* and 954063 and those from the crosses of P721*O* and IS-4404 (entries 6, 18, 81, and 149) had significantly lower levels of kafirin, ASG, and K + ASG than the parental lines 954063 and IS-4404.

Linear regression curves were plotted on the basis of these data for the relationships between percent kafirin, percent ASG, or percent K + ASG of total protein vs. lysine contents of the total proteins for all samples analyzed. The equation for percent lysine in protein vs. percent kafirin of total grain protein is % lysine = 4.78 − 0.0875 % K; the correlation coefficient, *r*, is −0.858; and the standard deviation is ±0.313. The relationship between percent lysine in protein vs. percent ASG of total grain protein is expressed by % lysine = 4.92–0.943 % ASG; the standard deviation is ±0.356; and the correlation coefficient is −0.816. The linear regression curve for percent lysine in protein vs. K + ASG percent of meal protein is shown in Figure 1. The expression for the equation is % lysine = 5.545 − 0.056 % K + ASG; the standard deviation is ±0.257; and the correlation coefficient is −0.894. The best inverse correlation between lysine content and alcohol–soluble protein extracted is to the K + ASG content of the grain protein. Simultaneous extraction of the two classes of alcohol-soluble proteins removes most efficiently a maximum of lysine-poor proteins that reduce grain lysine content.

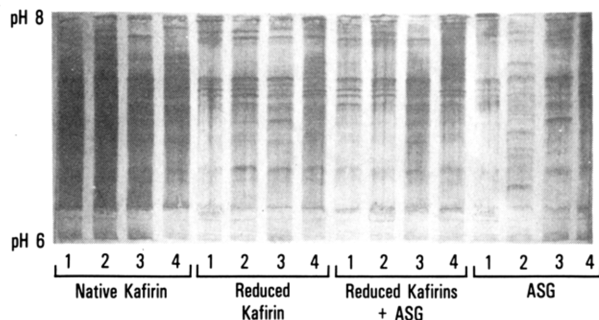

Figure 2. Isoelectric focusing patterns of native kafirins, reduced–alkylated kafirins, reduced–alkylated kafirins plus alcohol-soluble glutelin (K + ASG), and reduced alcohol-soluble glutelins (ASG). Extracts are from cultivars: (1) P721; (2) P721*O*; (3) IS-11758; (4) TE77.

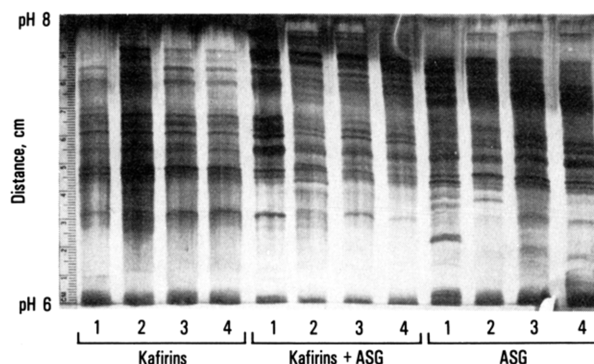

Figure 3. Isoelectric focusing patterns of reduced–alkylated kafirin, reduced–alkylated kafirins plus reduced–alkylated alcohol-soluble glutelin (ASG), and reduced–alkylated ASG from (1) P721*O*, vitreous sorghum 964063 (2), and inbred lines 94 (3) and 120 (4) from crosses of 964063 and P721*O*.

Isoelectric Focusing. In Figure 2, IEF patterns are shown for (left to right) directly extracted kafirins, reduced and alkylated kafirins, and ASG and K + ASG proteins for the four cultivars P721, P721*O*, IS-11758, and TE77. The native kafirins give streaked patterns, with a deficiency of bands appearing in the pH 6–7 range, as compared to the reduced alkylated proteins. Probably sorghum prolamins polymerize through disulfide interchange during dialysis and lyophilization, thus exhibiting different isoelectric points as compared to the polypeptides obtained after reduction and alkylation. The IEF patterns of reduced–alkylated kafirins from TE77, IS-11758, and P721 differ considerably (Figure 2). These results are consistent with the observations by Paulis and Wall (1979) that these three cultivars contain different alcohol-soluble proteins as evidenced by electrophoresis in aluminum lactate buffer at pH 3.1. In contrast the IEF pattern of kafirins of P721 and P721*O* are similar, which suggests that the *O* gene does not alter the relative expression of different genes coding for the kafirins but curtails production of all kafirins to a similar extent. The ASG from P721 has an IEF pattern similar to that of P721 kafirin, but the IEF pattern of ASG from P721*O* is different from that of P721*O* kafirin. It is possible that this ASG preparation was not properly reduced and alkylated as it resembles native kafirin; furthermore, in Figures 3 and 4 the IEF patterns of P721*O*

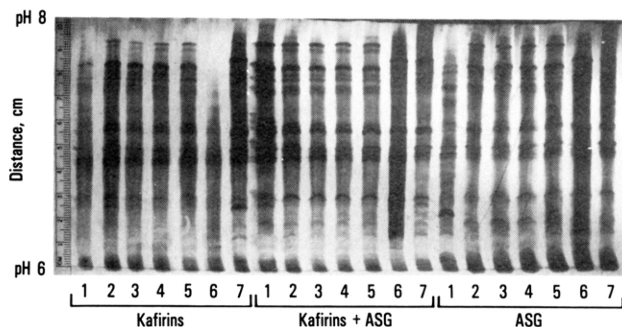

Figure 4. Isoelectric focusing patterns of reduced–alkylated kafirin, reduced–alkylated kafirin plus alcohol-soluble glutelin (ASG), and reduced–alkylated ASG from (1) P721*O*, inbred entries (2) 6, (3) 18, (4) 81, and (5) 149 derived from crosses of P721*O* and IS-4404 and (6) normal vitreous pigmented sorghum IS-4404 and (7) dehulled IS-4404.

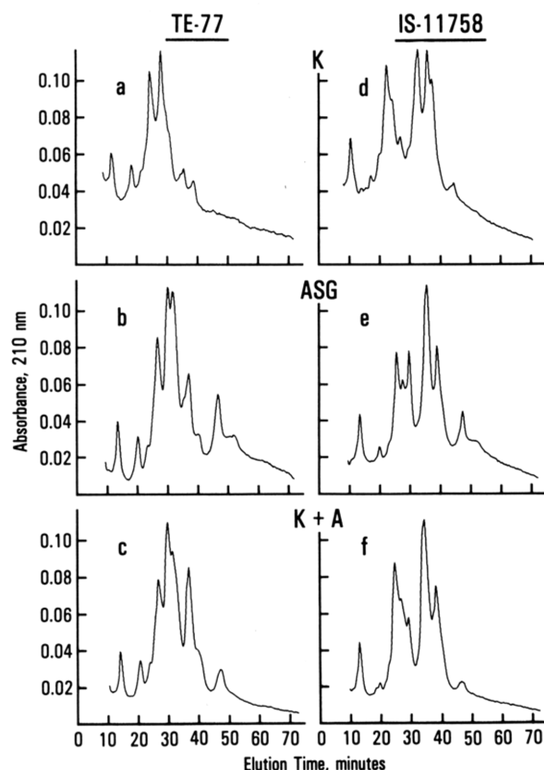

Figure 5. Reversed-phase high-performance liquid chromatography of reduced–alkylated kafirins (K), reduced–alkylated alcohol-soluble glutelins (ASG), and reduced–alkylated kafirins plus alcohol-soluble glutelins (K + ASG) extracted from hybrid TE77 (a–c) and Ethiopian high-lysine sorghum IS-11758 (d–f). Chromatographic conditions are described in text.

ASG appear similar to those of P721*O* kafirins. The IEF patterns of ASG polypeptides of the cultivars TE77, P721, and IS-11758 differ from one another, but each is similar to the corresponding IEF pattern of the kafirin from the same cultivar.

Isoelectric focusing patterns of P721*O* and inbred 954063 kafirins had a number of similar components but also exhibited significant differences (Figure 3). For example, prominent bands in P721*O* kafirin at 3.2 and 4.6 cm are absent in 954063 kafirin patterns, and bands at 4.0, 5.4, and 9.2 cm in 954063 kafirin's IEF separation are not present in that of P721*O*. Kafirins of the two inbreds, entries 94 and 120, derived from the cross of P721*O* and 954063, have similar IEF patterns (Figure 3). Many of their component proteins appear to be derived from the parental 954063 line, but some such as the bands at 8.4, 8.0, and 3.2 cm are unique to the P721*O* parent.

The IEF patterns of the ASG from 954063 also contained some components that differed from those of P721*O* ASG (Figure 3). The IEF patterns of ASG polypeptides from the inbreds 94 and 120 resemble each other. Both of these derived inbred ASG polypeptides had many components in common with the 954063 ASG, but each had some bands uniquely present in P721*O* ASG such as those at 2.0, 3.5, and 6.0 cm. Most of the separated proteins of ASG extracted from each of the four cultivars were also present in the IEF patterns of kafirins obtained from the same cultivar (Figure 3). However, there were quantitative and some qualitative differences between the components of the kafirin and ASG extracts for each cultivar. Some workers including Guiragossian et al. (1978) regard the ASG to be merely disulfide-crosslinked kafirin with compositional differences among polypeptide chains due to their differing tendency to form disulfide bonds. The single extracts of these sorghum grains containing both kafirin and ASG (K + ASG) had IEF patterns that were composites of those of the separate protein fractions. These K + ASG patterns allowed the best comparisons by IEF of the components of the alcohol-soluble proteins in the four cultivars. Bands at 3.2, 4.5, and 8.4 cm in the 94 and 120 inbred extracts appear derived from P721*O* while others were also present in 954063 storage protein or common to both extracts of both parents.

To further examine the effect of segregation and recombination during selection of inbreds for the *O* gene and yield factors from crosses of P721*O* and elite lines, the extracts from inbreds derived from the cross with IS-4404 were examined by IEF. IEF patterns of the alcohol-soluble proteins from P721*O*, derived inbred entries 6, 18, 81, and 149, and IS-4404 are illustrated in Figure 4. Patterns of alcohol-soluble proteins from the whole grain of IS-4404 are similar but less distinct than those from the dehulled samples, probably due to the effect of tannin complexing with and decreasing protein solubility (Figure 4). In the IEF patterns of P721*O*, IS-4404, and the derived inbred kafirins, a number of bands are common to extracts of all of these genotypes. However bands at 5.9, 6.5, 7.8, and 9.3 are present in patterns of kafirins from P721*O* and the derived inbreds but are absent in IS-4404 kafirin patterns. Bands at 5.5 and 7.5 cm in the IS-4404 kafirin pattern are absent in P721*O* and derived inbred kafirins IEF separations.

The IEF patterns of ASG polypeptides from the IS-4404 and from the inbreds derived from the cross of IS-4404 and P721*O* have many bands in common with the kafirins obtained from the same cultivar, although some qualitative and quantitative differences are evident, especially in the pH 6.5 (2.0 cm) and pH 7.5 (8.3 cm) regions of the gel (Figure 4). The P721*O* ASG has bands at 5.5, 7.0, and 8.2 cm that are absent in the ASG from IS-4404 but present in that of derived inbreds 6, 18, 81, and 149. In contrast, ASG bands at 2.5, 4.5, and 7.5 cm are common to extracts of IS-4404 and the derived inbreds but are not present in P721*O* ASG patterns. The K + ASG patterns again appear to be composites of the two separate fractions and establish that similar recombination and segregation of genes controlling alcohol-soluble proteins occurred during breeding in all four inbreds derived from the crosses of P721*O* and IS-4404.

High-Performance Liquid Chromatography of Sorghum Alcohol-Soluble Proteins. Reversed-phase high-performance liquid chromatography (RP-HPLC) offers an alternative method for separating alcohol-soluble proteins of sorghum grain; the resolution should be different since it depends on hydrophobic interaction rather

than charge. In Figure 5, HPLC chromatograms are compared for alcohol-soluble proteins extracted from kernels of the hybrid TE77 and the Ethiopian high-lysine sorghum IS-11758. The kafirins of TE77 elute in over eight peaks (Figure 5a). The TE77 ASG chromatogram (Figure 5b) contains the same protein peaks as that of the kafirin plus some other prominent later eluting, more hydrophobic components. The TE77 K + ASG extract (Figure 5c) yields an elution profile that approximates a composite of the patterns of the separately extracted kafirin and ASG proteins. Figure 5d illustrates the complex RP-HPLC elution pattern of the kafirins from IS-11758, and the separation of IS-11758 ASG is shown in Figure 5e. There are quantitative differences in heights of peaks eluting at the same positions in chromtograms of the two extracts. In addition, as in TE-77, some other more hydrophobic components are present in the ASG extract that elute late. The IS-11758 K + ASG single extract gives an elution profile (Figure 5f) that is a composite of both the separate chromatograms of the kafirins and of the ASG polypeptides when corrected for yields of both fractions. The elution patterns of the alcohol-soluble proteins of TE77 and IS-11758 are quite different and indicate genetic variation between the proteins of these two genotypes. Reversed-phase HPLC analyses were conducted on all of the kafirin, ASG, and K + ASG extracts from all of the genotypes examined in this study. For brevity of presentation, only data for the HPLC separation of K + ASG will be subsequently reported for the vitreous and opaque inbreds. These patterns reveal the genetic variation in proteins in these samples and are consistent with interpretations derived from separate kafirin and ASG HPLC elution patterns.

The differences between the TE77 and IS-11758 polypeptides constituting the K + ASG fractions (Figure 5) are apparently not related to the fact that the TE77 has normal endosperm while IS-11758 is a mutant high-lysine (*hl*) line. As shown in Figure 6 (parts a and b), elution patterns of K + ASG proteins from P721 and P721*O* sorghums are very similar, indicating that the mutation of the allele of the *O* gene does not affect structural genes that code for the synthesis of kafirin and ASG. The *O* gene only governs the amounts of kafirins and ASG proteins produced relative to albumin, globulin, and alcohol-insoluble glutelin proteins.

Parts b and c of Figure 6 allow comparison of RP-HPLC patterns for K + ASG proteins extracted from P721*O* and elite inbred 954063. Notable differences are evident; two moderate-sized peaks that occur in P721*O* K + ASG at 11- and 15-min elution times are replaced by smaller ones in the 954063 alcohol-soluble proteins. A large peak with a hump on its leading edge, indicating heterogeneity, at 25 min in P721*O* protein extract pattern is replaced by several smaller ones in 954063 proteins. A large peak in 954063 pattern at 30-min elution time is absent in that of P721*O*, and a large peak at 35 min elution time is replaced by a smaller peak with leading and trailing humps in P721*O*. The large peak at 43 min in the 954063 pattern is absent in that of P721*O* K + ASG. Elution diagrams in parts d and e of Figures 6 show proteins from two *Opaque* high-lysine inbreds, 94 and 120, derived from a cross between P721*O* and 954063. Characteristic peaks from P721*O* extract at 11-, 15-, and 25-min elution times are also present in K + ASG of the two inbreds. Peaks at 30, 35, and 43 min in the elution diagram of K + ASG from 954063 are present in the protein patterns of inbred 94 (Figure 6d). Inbred 120 K + ASG has peaks (Figure 6e) resembling those from 954063 at 30 and 43 min but lacks a major peak

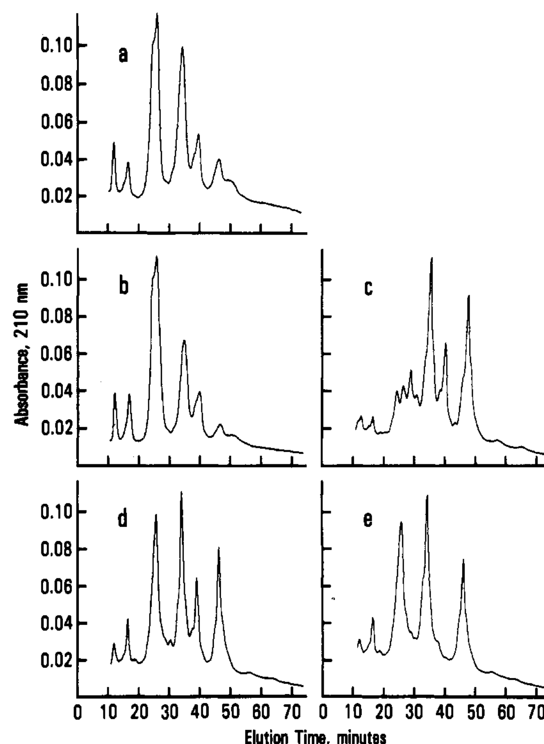

Figure 6. Reversed-phase high-performance liquid chromatography elution profiles of reduced–alkylated kafirins plus alcohol-soluble glutelins (K + ASG) from normal and opaque high-lysine sorghums: (a) inbred P-721 (normal line); (b) P721*O* (opaque mutant); (c) inbred 964063 (normal vitreous parent); (d) inbred 94; (e) inbred 120 (derived from crosses of 964063 and P721*O*). Column and solvent gradient as given in text.

at 35 min. These results suggest that K + ASG proteins are coded on at least two different chromosomes. The slower moving, more hydrophobic components present in peaks at 35 and 43 min seem to be associated mainly with ASG, while the early peaks are mainly in kafirin extracts.

To further test the hypothesis that more than one chromosome is involved in coding for K + ASG proteins, the second series of inbreds derived from a cross between P271*O* and inbred IS-4404 was investigated by RP-HPLC (Figure 7). The pattern of the K + ASG extract from P721*O* (Figure 7a) differs slightly from that in Figure 6b since a different column was used for the two different series of separations. The elution patterns of the K + ASG protein fraction from P721*O* and IS-4404 (Figure 7, parts a and b, respectively) differ significantly. P721*O* K + ASG has small peaks at 20 and 24 min, a major peak at 33-min elution time, and peaks at 43 and 47 min. Inbred IS-4404 alcohol-soluble proteins have a moderate peak at 38 min and others at 46 and 60 min.

The patterns of the four opaque inbreds (entries 6, 18, 81, and 149; Figure 7, parts c–f, respectively) selected from progeny of the P721*O* X IS-4404 cross have very similar K + ASG elution patterns. The first three peaks (at 20, 24, and 35 min) are identical with those in P721*O*. Among the three peaks eluting between 42 and 48 min, the first and third appear to be derived from P721*O* while the middle and most prominent one resembles the peak eluting at 46 min in the chromatographic pattern of IS-4404 K + ASG. Thus, it again appears that segregation and recombination of chromosomes have occurred in the opaque progeny of the cross of P721*O* and IS-4404. The earlier eluting peaks appear to be derived mainly from P721*O* and the later ones from IS-4404. Since these earlier peaks appeared in the K + ASG extracts from inbreds derived from crosses of P721*O* with both 954063 and IS-4404, it

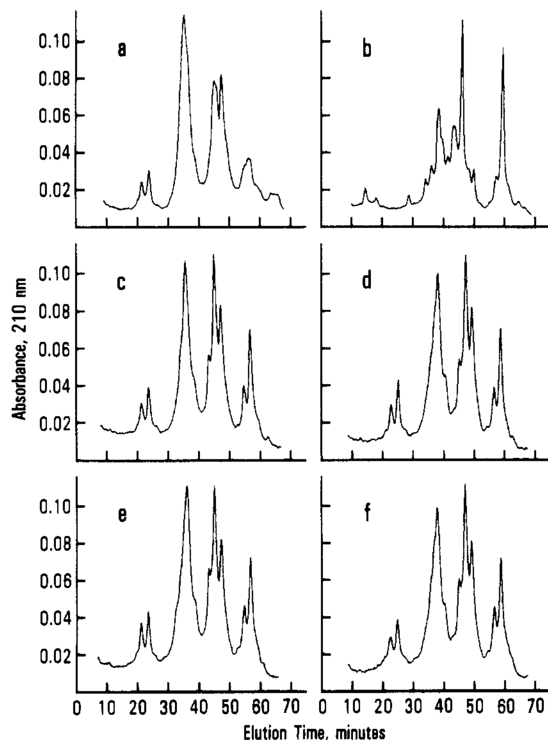

Figure 7. Reversed-phase high-performance liquid chromatography profiles of reduced–alkylated kafirins plus alcohol-soluble glutelins (K + ASG) from normal and opaque high-lysine sorghums: (a) P721*O*; (b) IS-4404, inbred entries; (c) 6; (d) 18; (e) 81; (f) 149 derived from crosses of P721*O* and IS-4404. Chromatographic conditions are given in the text.

is possible that the *O* gene is on the same chromosome as genes coding for the more hydrophilic proteins in the K + ASG complex.

DISCUSSION

In agreement with other investigations (Guirogossian et al., 1978; Paulis and Wall, 1979) the results of this study indicate that the lysine level of sorghum grain decreases with an increase in the amount of kafirin and ASG. High-lysine sorghums have reduced levels of these two classes of proteins. Due to the positive or negative effect of tannins and other pigments, quantitative extraction of the kafirins and ASG was not achieved in certain cultivars of sorghum. In corn an inverse linear correlation was established between the amount of zein plus alcohol-soluble glutelin and the lysine content of the grain protein (Paulis and Wall, 1975). In this study the correlation coefficient between lysine and kafirins was −0.86 and between lysine and ASG, −0.82. The correlation coefficient between lysine and K + ASG is better, −0.89, indicating that variations in both sorghum alcohol-soluble proteins influence lysine content and that the higher extraction of K + ASG would allow a better estimation of lysine from a regression curve. In Figure 1, the regression curve of K + ASG vs. lysine for all genotypes in this study, similar values of lysine and K + ASG in the high-lysine lines and similar values in the vitreous grains result in two separate clusters, suggesting both sorghum *hl* and *O* genes effect similar biochemical processes.

Like our previous study using polyacrylamide gel electrophoresis with an acid buffer in 8 M urea (Paulis and Wall, 1979), the present studies using IEF and RP-HPLC have demonstrated that the compositions of alcohol-soluble proteins from near-isogenic lines of normal- and high-lysine sorghums (P721 and P721*O*) are qualitatively very similar. Since several kafirin or ASG proteins may have the same isoelectric point, IEF separations alone may result in anomalous conclusions. However because reversed-phase HPLC separates by a different process, hydrophobic adsorption, the two methods supplement one another to provide better support for our findings. Evidently both normal- and high-lysine sorghums in isogenic lines have similar families of structural genes coding for their storage proteins. These multigene families vary with the genetic background of the sorghum cultivars so that most inbred lines of sorghum exhibit different IEF or HPLC separation patterns for their kafirin and ASG proteins. Alleles at the same gene loci coding for K and ASG are codominant, and the proteins from both parents are expressed in hybrids (Sastry et al., 1986). Upon recombination and segregation in the F_2 generation, except for crossing over or mutation, genes carried on a single chromosome will be transferred together and expressed in the composition of the endosperm storage protein. Several successive generations of these F_2 selections were selfed to ensure homozygous inbred lines. The occurrence of some of these proteins together in several inbreds derived from F_2 generations indicates that the genes coding for these proteins are closely linked on a single chromosome. Since some proteins are transmitted from both parental lines it appears that the storage proteins are coded on two or three different chromosomes. There is evidence that structural genes coding for zeins of closely related corn are located on three chromosomes, 4, 7, and 10 (Soave and Salamini, 1984).

In corn the *opaque 2* (*o2*) mutation located on chromosome 7 reduces the content of only certain of the zein proteins. Of the two major zein molecular weight species, 20 and 22 kDa, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) separations by Lee et al. (1976) indicate that the 22-kDa component was decreased in *o2* endosperm. Soave and Salamini (1984) related these proteins to specific zein bands separated by IEF and coded by genes on corn chromosome 4. The *floury 2* mutant gene also reduces zein content in corn endosperm but appears to diminish both 20 and 22 kDa zeins to the same extent. Paulis and Wall (1979) observed no differences in intensities of the major bands of kafirins and ASG polypeptides separated by molecular size by SDS-PAGE. In the present studies no differential inhibitory effect by the *O* gene on production of specific kafirins or ASG was observed. This finding suggested that the *hl* and *O* genes of sorghum curtail production of all kafirin and ASG proteins to the same extent.

Examination of IEF patterns of K + ASG proteins from the opaque inbreds derived from the crosses of P721*O* with both 954063 and IS-4404 establishes that their protein profiles all contain the same proteins derived from P721*O*. In the IEF patterns of the derived inbreds, some bands resemble the proteins present in extracts of the normal elite parent of the hybrid. The RP-HPLC elution diagrams of K + ASG of the derived inbreds clearly illustrate that one group of proteins appears to be present in all of the opaque lines. These more hydrophilic proteins may be closely linked to the *O* gene on a single chromosome and are derived from P721*O*. The other alcohol-soluble proteins in the grains resemble some of those from either of the normal elite parents of the hybrids. Selection pressure to improve yield may have resulted in retention of chromosomes from the elite parent. Unfortunately at this time the identities of the specific chromosomes on which the K and ASG proteins are coded and the *O* gene is found are not known. Location of these genes on sorghum chromosomes and information as to their proximity would be of considerable interest. The manner in which the *O*

gene reduces the expression of the kafirin and ASG genes would be valuable information to better understand the linkage between the soft-kernel characteristics and the improved protein quality of the P721O line and of the higher yielding lines derived from its crosses with elite inbreds.

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LITERATURE CITED

Butler, L. G.; Riedl, D. J.; Lebryk, D. G.; Blytt, H. J. *J. Am. Oil Chem. Soc.* **1984**, *61*, 916.
Guiragossian, V. Y.; Chibber, A. K.; Van Scoyoc, S.; Jambunathan, R.; Mertz, E. T.; Axtell, J. D. *J. Agric. Food Chem.* **1978**, *26*, 219.
Jambunathan, R.; Mertz, E. T.; Axtell, J. D. *Cereal Chem.* **1975**, *52*, 120.
Lee, K. H.; Jones, R. A.; Dalby, A.; Tsai, C. Y. *Biochem. Genet.* **1976**, *14*, 641.
Mohan, D. D.; Axtell, J. D. Proceedings 9th Annual Grain Sorghum Research and Utilization Conference, Lubbock, TX, March 4-6, 1975; p 85.
Paulis, J. W.; Wall, J. S. In *Protein Nutritional Quality of Foods and Feeds, Part I, Assay Methods; Biochemical and Chemical*; Friedman, M., Ed.; Marcel Dekker: New York, 1975; p 381.
Paulis, J. W.; Wall, J. S. *J. Agric. Food Chem.* **1977**, *25*, 265.
Paulis, J. W.; Wall, J. S. *Cereal Chem.* **1979**, *56*, 20.
Sastry, L. V. S.; Paulis, J. W.; Bietz, J. A.; Wall, J. S.; Ross, W. M. *Cereal Chem.* **1986**, in press.
Singh, R.; Axtell, J. D. *Crop Sci.* **1973**, *16*, 296.
Soave, C; Salamini, F. *Philos. Trans. R. Soc. London, B* **1984**, *304*, 341.
Van Scoyoc, S. W. Thesis, Purdue University, West Lafayette, IN, 1979.
Wall, J. S.; Paulis, J. W. *Advances* in *Cereal* Science *and Technology II*; Pomeranz, Y., Ed.; American Association of Cereal Chemists: St. Paul, MN, 1978; p 135.
Wall, J. S.; Fey, D. A.; Paulis, J. W.; Landry, J. *Cereal Chem.* **1984**, *61*, 141.

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Cadmium, Lead, Mercury, and Arsenic Concentrations in Crops and Corresponding Soils in The Netherlands

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Cereals, potatoes, vegetables, fruits, and fodder crops were collected from their major growing areas in The Netherlands and were analyzed, together with their corresponding soils, for Cd and Pb and partly for Hg and As. After a large number of samples per crop were collected, the base-line levels could be determined. The Cd and Pb levels of cereals were high with respect to the proposed maximum acceptable concentrations for human consumption in The Netherlands. In lettuce and spinach relatively high Cd levels occurred, and in fruits such as tomatoes, cucumbers, and apples Cd levels were low. The Pb level in curly kale was high. The soils had median values for Cd, Pb, Hg, and As of 0.4, 23, 0.07, and 11 mg/kg of dry soil, respectively; the levels of the greenhouse soils were somewhat higher for Cd, Pb, and Hg. In the case of high metal concentrations in a crop the source of contamination could sometimes be indicated.

INTRODUCTION

There is international concern about human intake of toxic trace elements such as cadmium, lead, mercury, and arsenic. Intake of relatively low doses of these elements over a long period can lead to malfunction of organs and chronic toxicity. Toxic trace elements are in part ingested with the edible parts of agricultural and horticultural crops or derived products. The determination of the base-line levels of Cd, Pb, Hg, and As in agricultural and horticultural crops is necessary to evaluate their toxicological significance and to set action levels.

In a number of countries a survey of Cd, Pb, and Hg in various crops has been carried out. Extensive studies were reported from Sweden (Fuchs et al., 1976), Denmark (Hansen and Andersen, 1982), and Finland (Koivistoinen, 1980). Between 1971 and 1975 several publications appeared in Great Britain, each concerning a different metal (Ministry of Agriculture, Fisheries and Food, 1971, 1972, 1973, 1975). In Germany vegetables and fruits were bought at retail markets and analyzed for Cd, Pb, Hg, and As (Barudi and Bielig, 1980). The major agricultural crops of the U.S. were also analyzed for cadmium and lead (Wolnik et al., 1983, 1985). Besides these extensive studies, in several countries one or more crops were analyzed for

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