AMINO ACID COMPOSITION AND STORAGE PROTEINS IN TWO NEW HIGH-LYSINE MUTANTS IN MAIZE[1,2,3]

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ABSTRACT

Two newly identified high-lysine mutants of maize, *opaque*-6 (o_6) and *floury*-3 (fl_3), have been compared with the appropriate nonmutant controls for their effect on endosperm proteins and amino acid content. Similar data are also presented for the high-lysine mutant *opaque*-7 (o_7) and two opaque mutants, *opaque*-1 (*o*) and *soft-starch* (*h*) that do not have altered amino acid compositions. The o_6 and fl_3 mutants, in spite of increased lysine content, do not lend themselves to practical applications. Albumins and nonprotein nitrogen, globulins, prolamines, and glutelins were extracted sequentially by a modified Osborne-Mendel procedure. Significant differences among the mutant strains were found in regard to protein distribution patterns. Three mutants, o_6, o_7, and fl_3, which are relatively high in lysine content, showed a shift in zein:glutelin ratio as well as an increase in nonprotein nitrogen, albumins, globulins (except fl_3), and insoluble proteins. The two mutants with normal lysine contents, *o* and *h*, showed a protein distribution pattern similar to the normal strains. Amino acid analyses of the three high-lysine mutant endosperms and the isolated protein fractions, together with the protein fractionation data, show that the increase of lysine in these three mutants resulted from the decrease in the ratio of zein to other protein fractions.

Mutant genes that effect substantial changes in the amino acid content of the collective endosperm proteins have now been isolated in maize (1,2,3), barley (4,5), and sorghum (6). In the *opaque*-2 mutant of maize, the basis of the altered amino acid pattern of the endosperm is a change in the proportion of the proteins normally present (1,7,8,9). The alcohol-soluble prolamine fraction (zein) is much reduced, and the water-soluble proteins (albumins), the salt-soluble proteins (globulins), and the free amino acids are markedly enhanced. The same changes have been noted in the *floury*-2 mutant (7,10), the *opaque*-7 (11) mutants of maize, the *Risφ* 1508 mutant in barley (5), and the h_1 mutant of sorghum (12). The only mutant with an altered amino acid content that does not correspond to this pattern is the *hiproly* mutant of barley (13). In this mutant, the amount of the alcohol-soluble protein fraction is decreased slightly while the amounts of four lysine-rich, water-soluble proteins are increased greatly.

[1]Part of a thesis submitted by the first author in partial fulfillment of the requirement for the M.S. degree.

[2]Research supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and by National Institutes of Health Grant 15422.

[3]Laboratory of Genetics Paper No. 1792.

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To date, investigations of the mutants with altered amino acid balance in maize, barley, and sorghum have borne out the hypothesis that marked changes in the amino acid content of cereal seeds will arise only by mutations that effect changes in the proportions of proteins synthesized (14).

We report here on the protein fractionation of two new maize mutants, *opaque*-6 (o_6) and *floury*-3 (fl_3) that also condition altered amino acid profiles similar to those found in *opaque*-2 and *floury*-2 seeds.

The *opaque*-6 and *floury*-3 mutants cannot be utilized to produce maize of superior protein quality since o_6/o_6 plants die as seedlings, and homozygous *floury*-3 seeds are very light in comparison to normal seeds. Additionally, in some genetic backgrounds, fl_3/fl_3 seeds will not germinate. Nevertheless, examinations of these mutants constitute additional tests of the hypothesis that major shifts in amino acid contents occur only when synthesis of the alcohol-soluble proteins is suppressed to some extent, and compensatory synthesis of other fractions results.

Additionally, we report an investigation of the *opaque*-7 mutant of maize which has been analyzed by other laboratories (3,11).

No investigations of the protein fractions of the *o* or *h* mutants (2) of maize have been reported. The simplest expectation is that the proportions of the various endosperm proteins would not be altered. However, such changes not reflected in alterations in the overall amino acid composition of endosperm proteins could conceivably occur and form the basis of the opaque phenotype by which these mutations were detected. We report here the results of analyzing these mutants.

MATERIALS AND METHODS

The materials used for these studies were mature kernels of five mutant strains, *opaque*-1 (*o*), *opaque*-6 (o_6), *opaque*-7 (o_7), *soft starch* (*h*), *floury*-3 (fl_3), with three normal entries which were nonmutant counterparts of o_6, o_7, and fl_3, respectively.

The o_6 mutant was received from R. B. Ashman in 1968. This recessive mutation was found in popcorn and was placed in a dent background. The o_6/o_6 plants die as seedlings, but mutant kernels can be obtained by self-pollinating plants grown from phenotypically normal heterozygous kernels. Additionally, the phenotypically normal kernels on such ears provided the nonmutant counterpart with which the mutant kernels are compared.

The *opaque*-7 mutant which occurred in the inbred line W22 was received from K. S. McWhirter in 1971. The seeds analyzed were grown here in 1971. The W22 seeds used for comparison were grown in 1971 also.

The *floury*-3 mutation was obtained from Bear Seed Company in 1968. It has been designated as *floury*-3 since two doses of the mutant gene in the endosperm condition a mutant phenotype as do fl_1 and fl_2. The mutant and the normal seeds used were obtained by self-pollinating $+/fl_3$ plants. Tests of allelism showed that *floury*-3 is not allelic to any of the known opaque or floury mutants. Tests with a series of *waxy* translocation stocks indicate that fl_3 is probably located on chromosome 8.

In this paper, we use the gene symbol (*e.g.*, o_6) to refer to endosperms which are homozygous for the mutant gene under discussion (*e.g.*, $o_6/o_6/o_6$). The

exception to this usage is the *floury*-3 mutant which in two doses conditions a mutant endosperm phenotype. Thus, the symbol fl_3 indicates kernels that are phenotypically *floury*-3 and may be either $fl_3/fl_3/+$ or $fl_3/fl_3/fl_3$.

The mature kernels were soaked in water for 1 to 2 hr. The pericarps and embryos were completely removed from the endosperm. The endosperms were dried for 48 hr at 40° C, ground in a Wiley Mill to pass a 60-mesh screen, and defatted for 36 hr with n-hexane in a Soxhlet apparatus.

Fractionation

The endosperm protein was fractionated by successive extractions using the solvents which yield the four Osborne-Mendel fractions: water-soluble proteins or albumins plus the free amino acids; salt-soluble proteins or globulins; ethanol-soluble proteins or prolamines (zein); and alkali-soluble proteins or glutelins. Ten grams of defatted endosperm powder was extracted with the following solvents in sequence: water, 5% sodium chloride solution, 70% ethanol, and 0.2% sodium hydroxide solution to yield the albumins, globulins, prolamines, and glutelins, respectively. The extraction methods were those used by Jimenez (15), except that 15 ml of solvent was used for each g of powder. The proteins in the water extract were precipitated by phosphotungstate (1 part of 1% phosphotungstate to 4 parts of the water extract) to allow a determination of content of free amino acids and small peptides. The sodium hydroxide extract was titrated to pH 6 with 10% acetic acid. The protein that precipitated at pH 6 is referred to as the pellet and that remaining in solution as the supernatant fraction.

Nitrogen Determination

The nitrogen content of the whole defatted endosperm and of each of the recovered fractions was determined by the micro-Kjeldahl method described in

TABLE I
The Distribution of Nitrogenous Fractions Expressed as mg/Endosperm

Fraction	Genotype							
	+/−[a]	o_6	W22	o_7	+/−[b]	fl_3	*o*	*h*
H_2O-soluble	0.49	2.27	0.67	2.55	1.23	1.95	0.84	0.90
Albumins	0.43	1.42	0.52	1.49	0.89	1.40	0.69	0.75
Free acids	0.06	0.82	0.15	1.06	0.34	0.55	0.15	0.15
Globulins	0.43	0.66	0.39	0.55	0.71	0.30	0.69	0.44
Prolamine	9.59	2.91	7.83	1.64	13.7	4.83	12.75	6.91
Glutelins	4.56	3.81	6.00	4.93	8.09	4.85	7.89	5.14
Supernatant	1.06	0.77	1.57	0.98	1.87	0.96	1.70	0.90
Pellet	3.50	3.06	4.43	3.95	6.22	3.89	6.19	4.24
Residue	0.29	1.29	0.66	0.84	0.91	0.71	0.82	0.31
Actual recovery	98.0	95.3	99.8	95.7	95.8	101.4	101.7	101.3
Total protein (g)								
10 g Endosperm	1.22	1.07	1.05	0.89	1.20	1.33	1.22	1.02
No. endosperms/10 g	78.1	93.2	67.1	81.2	46.8	106.7	54.1	75.8

[a]The normal counterpart of o_6.
[b]The normal counterpart of fl_3.

detail in AOAC (16) with some modifications. The nitrogen content was multiplied by a constant—6.25—to obtain the protein content.

Amino Acid Analysis

Acid hydrolysis was used for all samples and norleucine was added as an internal standard (1). All the analytical work was carried out by means of a Technicon automatic amino acid analyzer.

RESULTS AND DISCUSSION

Protein Fractionation

Variations were found among the five mutants in regard to the content of zein as well as other proteins (Table I).

Three mutants, o_6, o_7, and fl_3 showed a shift in the zein:glutelin ratio from that measured for their normal controls. It should be noted, however, that the total amount of glutelin per endosperm for the mutants is lower than that in the normal endosperms. This contrasts with *opaque*-2 where the glutelin content per endosperm is equal to nonmutant controls (15). The total water-soluble fractions in these three mutants had the highest increase in nitrogen concentration among all fractions. In addition, the insoluble-protein fractions were also high in these three mutants. The globulin contents of o_6 and o_7 relative to nonmutant counterparts were increased. This was not the case, however, with fl_3.

On the other hand, mutants *o* and *h* were similar to the three phenotypically normal strains in i) the zein: glutelin ratio; and ii) the content of each of the other protein fractions. The percentage value of zein among the total proteins in the present study is similar to the result obtained by Wolf *et al.* (17).

In the present study, we report slightly different results from Misra *et al.* (11) for the fractionation of *opaque*-7 endosperm proteins. This difference probably has its basis in the modified Osborne-Mendel procedure used in the present paper, whereas extraction sequence D of Landry and Moureaux (18) was used by Misra *et al.* (11). Also, they were assaying seeds grown in 1970 in Australia, and environmental influences may complicate the comparison. Their results showed that the saline-soluble fraction (including free amino acids, albumins, and globulins) was 16.6% of *opaque*-7 endosperm nitrogen which was lower than the sum (29.5%) of the water- and salt-soluble fractions in the present report. A slightly higher zein content was also reported in their work.

Amino Acid Analysis

The amino acid analysis of whole endosperms is given in Table II. Among the mutants, o_6 had the highest lysine concentration, and o_7, fl_3, *h*, and *o* followed in order. The amino acid compositions of the *o* and *h* mutants were quite comparable to the composition of the nonmutant kernels analyzed although the lysine content of *h* is somewhat higher in this analysis. A previously reported analysis of *h* (2) gave a lysine content of 1.8.

The overall amino acid compositions of mutant o_6 and o_7 endosperm proteins were rather similar to those of the *opaque*-2 and *floury*-2 mutants. In addition to the elevated lysine content, one observes a characteristic increase in arginine, aspartate, and glycine, and a decrease in proline, alanine, and leucine as compared to normal. The analysis of fl_3 proteins showed the characteristic

increases and decreases noted above but generally to a lesser extent.

Amino acid analyses of the protein fractions were performed only on the higher-lysine mutants, *o6*, *o7*, and *fl3* and their nonmutant controls. The lysine concentrations in each protein fraction of these six genotypes are given in Table III. The lysine contents of the prolamine and residue fractions do not vary markedly from mutant to normal within any comparison. However, the lysine contents of the water-soluble, globulin, and glutelin fractions of *o6*, the water-soluble fraction of *fl3*, and the prolamine and glutelin fractions of *o7* are higher than the contents of the corresponding fractions from the appropriate controls. It is not clear, however, in the absence of replicated extractions and replicated amino acid analyses that these differences are meaningful. A change in lysine content in a solubility fraction of a mutant is not unprecedented since Jimenez (7) has reported an increased lysine content of the glutelin fraction of *o2* as compared to normal. Such changes probably have their basis in the changed proportions of the various proteins constituting the fraction since Jimenez was unable to demonstrate any missing proteins or proteins altered in electrophoretic mobility in starch gel-urea electrophoresis. Thus, the observed alterations in the overall amino acid composition of the collective endosperm proteins from such mutants as *o2*, *o6*, and *o7* derive not only from the changed proportions of the solubility fractions but probably also from the changed proportions of the proteins within the fractions. This change of protein proportions within a fraction has already been noted as the basis of the elevated lysine content in the *hiproly* mutant of barley (13).

TABLE II
Amino Acids in the Endosperms of *o6*, *o7*, *fl3*, and their Nonmutant Counterparts, *o* and *h* Expressed as mg per 100 mg Protein

	Genotype							
Amino Acids	+/−[a]	*o6*	**W22**	*o7*	+/−[b]	*fl3*	*o*	*h*
Lysine	1.55	3.32	1.78	3.24	1.59	2.68	1.62	2.21
Histidine	2.93	3.00	3.37	3.53	3.54	4.08	3.14	2.88
Arginine	2.98	3.93	2.85	3.27	2.75	3.15	2.96	3.22
Aspartic acid	5.74	9.80	6.11	12.13	5.92	7.22	5.94	6.48
Threonine	3.48	3.65	3.67	3.95	3.49	3.69	3.33	3.64
Serine	5.12	4.78	5.09	4.82	5.42	5.11	5.10	5.12
Glutamic acid	20.54	22.95	20.62	23.23	20.43	20.03	20.97	20.21
Proline	10.59	7.09	10.85	8.81	10.24	10.12	10.19	10.08
Glycine	2.54	3.38	2.97	3.44	2.49	3.30	2.54	3.10
Alanine	8.24	7.38	7.79	7.09	8.12	7.59	8.32	7.95
Valine	3.23	3.23	3.32	3.19	3.19	3.22	2.94	3.30
Methionine	2.55	3.18	2.63	2.60	2.04	1.67	2.53	2.96
Isoleucine	3.92	3.62	3.64	3.22	3.76	3.81	3.79	3.73
Leucine	15.96	11.76	15.27	10.67	15.91	14.23	16.26	14.87
Tyrosine	4.12	3.80	3.75	2.13	4.42	3.72	3.71	4.13
Phenylalanine	6.51	5.13	6.11	4.68	6.78	6.37	6.68	6.13
Data corrected to 100% recovery of protein								
Actual recovery	101.9	91.5	106.6	104.9	97.4	110.4	100.9	100.3

[a]The nonmutant counterpart of *o6*-R.
[b]The nonmutant counterpart of *fl3*.

TABLE III
The Lysine Content in the Protein Fractions of o_6, o_7, fl_3 and their Normal Counterparts Expressed as (a) mg/100 mg Protein, (b) μg/Endosperm, and (c) Percentage of Total Lysine Recovered

Fraction		+/−[a]	o_6	W22	o_7	+/−[b]	fl_3
H_2O-soluble	a	4.82	6.13	3.75	4.00	4.80	6.48
	b	24.1	146.0	25.2	106.7	61.7	126.2
	c	11.6	35.2	9.4	29.9	13.0	36.2
Albumins	a	4.55	5.81	4.08	5.36	4.28	6.21
	b	19.3	82.5	21.2	80.3	37.7	87.2
	c	9.3	19.9	7.9	22.5	7.9	25.0
Nonprotein nitrogen	a	8.25	7.56	2.72	2.49	7.04	6.61
	b	4.7	63.4	4.0	26.4	24.0	39.0
	c	2.3	15.3	1.5	7.4	5.1	11.2
Globulins	a	6.08	6.66	5.47	6.02	5.91	5.60
	b	26.1	43.1	21.3	32.8	41.7	16.8
	c	12.6	10.4	8.0	9.2	8.8	4.8
Prolamines	a	0.16	0.06	0.09	0.11	0.42	0.22
	b	15.2	1.6	7.0	1.8	60.0	10.6
	c	7.3	0.4	2.6	0.5	12.6	3.1
Glutelins	a	2.81	4.07	3.20	3.72	3.15	3.26
	b	130.8	164.2	192.0	192.3	266.5	158.2
	c	62.9	39.5	71.8	53.9	56.1	45.5
pH 6 Soluble	a	1.15	3.92	1.28	1.99	1.07	3.15
	b	12.1	31.2	20.0	19.2	19.9	30.2
	c	5.8	7.3	7.5	5.4	4.2	8.7
pH 6 Insoluble	a	3.40	4.37	3.89	4.35	3.98	3.37
	b	118.7	133.4	172.0	173.1	246.6	128.0
	c	57.1	32.2	64.3	48.5	51.9	36.8
Residue	a	4.12	4.69	3.31	2.74	4.99	5.02
	b	11.7	60.1	21.8	22.8	45.3	35.6
	c	5.6	14.5	8.2	6.4	9.5	10.2
Recovered	b	207.9	415.0	267.9	356.4	475.2	347.4
Total	b	240.9	387.5	346.6	356.1	407.7	354.6

[a]The nonmutant counterpart of o_6 (+/+/+, +/+/o_6, and o_6/o_6/+).
[b]The normal counterpart of fl_3 (+/+/+ and +/+/fl_3).

Table III also demonstrates that the major portion of the enhanced lysine content in the mutants was contributed by the water-soluble fraction. Our results for o_6 and o_7 agree with those of Jimenez (15) for o_2 and fl_2 in that more lysine is contributed by the glutelin fraction than any other single fraction. However, for o_6 and o_7, we find a relatively lower contribution to lysine content by the glutelin fraction and a higher contribution by the water-soluble fraction than was estimated for o_2 and fl_2. Among all fractions in normal endosperms, zein had the

lowest lysine content even though it supplied more than 50% of the total endosperm proteins.

It has been pointed out that the nonmutant counterpart of fl_3 kernels may be either $+/+/fl_3$ or $+/+/+$. The fact that 0.5 of the nonmutant kernels is carrying one dose of the fl_3 allele may account for our observation that this class has a much higher content of lysine per kernel in the water-soluble fraction (Table III) than do the two other nonmutant entries analyzed. It is not sufficiently high, however, to have a discernible effect on the lysine content of the bulked endosperm proteins (Table II).

DISCUSSION

For the *o* and *h* mutants which were previously known not to alter amino acid composition, it is clear that no marked changes are observed in the proportions of the storage proteins as compared to the nonmutant entries. For the three mutants with altered amino acid compositions, (o_6, o_7, and fl_3) the changes in the storage protein proportions are clear. The fl_3 mutant which shows the least deviation from normal maize in overall amino acid composition also shows the least deviation from normal with respect to the proportions of the various classes of storage proteins.

Thus for the o_2, o_6, o_7, fl_2, and fl_3 mutants of maize, the *hiproly* and *Risφ* 1508 mutants of barley, and the *hl* mutant of sorghum, alterations in amino acid composition are associated with and probably a consequence of altered proportions of normal storage proteins and of some alteration in the composition of the albumin and glutelin fractions. This has been suggested previously as the only route by which such alterations could occur (14). No mutant has yet been detected in the cereals in which an altered amino acid composition is the result of the synthesis of a protein or proteins with altered primary sequences, nor has any mutant been detected in which the content of only one amino acid is altered.

The inviability of o_6/o_6 plants raises an interesting problem. Table I indicates a pronounced change in the proportions of the storage proteins accounting for a substantially altered amino acid composition (Table II) which is much like that of the *opaque*-2 mutant (2). The storage proteins of cereal seeds presumably serve only as a source of readily available nitrogen for the germinating seed. On this basis, the lethality of o_6/o_6 plants is difficult to explain since the shift in the proportions of storage proteins is similar to that observed in *opaque*-2, *opaque*-7, and *floury*-2 which are viable. In *opaque*-6, we may have an example of a mutation affecting a function vital for the sporophytic generation but not for endosperm development. However, the lack of the function during endosperm development conditions the shift in proportions of the proteins synthesized. Alternatively, it is possible that the mutation in changing the proportion of solubility fractions being synthesized (and the proportion of various proteins within each function) may affect the synthesis of an albumin or globulin component which is essential during the early stages of plant growth but is not synthesized *de novo* upon germination.

Acknowledgment

We thank Dr. Oliver Smithies for making available his amino acid analyzer for our use.

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[Received July 15, 1974. Accepted October 29, 1974.]