ENDOSPERM PROTEIN FORMATION DURING KERNEL DEVELOPMENT OF WILD TYPE AND A HIGH-LYSINE BARLEY MUTANT[1]

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Cereal Chemistry 53(6): 890-901

ABSTRACT

The formation of barley endosperm proteins was followed by amino acid analysis and SDS-polyacrylamide gel electrophoresis during kernel development of wild type barley and the high-lysine mutant, Risø No. 1508. In the mutant, only minor differences in the amino acid composition and electrophoretic patterns of the albumins and the globulins, respectively, were observed, but three times more free amino acids were present at all stages of endosperm development compared to the wild type. Hordein formation was severely impaired and the synthesis of two components of the fraction was inhibited in the mutant. The high-lysine content of the mutant endosperm is due to this reduction in the lysine-poor hordeins, a reduction of lysine-poor components of the glutelin fraction, and a compensating increase in the amount of lysine-rich glutelins as well as free lysine.

Several genes are known to alter the overall amino acid composition of mature barley seeds (1,2). A mutation in the cultivar Bomi (3,4) gave rise to the mutant Risø No. 1508, in which the lysine content of the mature kernels is increased by 45%. This high-lysine content is a consequence of a reduction in the amount of lysine-poor hordeins and an increase in free amino acids (3,5). The mutant endosperm contains protein bodies of a structure entirely different from that found in the Bomi barley endosperm (6), and hordeins are only poorly represented in the mutant protein body (7).

At early stages of barley grain development, Bishop (8) found accumulation of the salt-soluble proteins, whereas the accumulation of hordeins occurred later in the maturation period. Smith (9) and Pomeranz and Robbins (10) showed that during maturation glutamic acid, proline, and cysteine increased significantly with a compensating decrease in alanine, lysine, aspartic acid, and threonine. Ivanko (11) showed that these changes were mainly the result of an increase in the concentration of hordeins, which have high concentrations of glutamic acid and proline and low concentrations of aspartic acid, alanine, and lysine.

Few comparative studies on high-lysine and normal barley endosperm proteins during kernel development have been published. Munck (12) compared nitrogen accumulation in modified Osborne fractions in Hiproly and its normal counterpart CI 4362 during kernel development, and found minor differences in the deposition of hordeins and in the total deposition of nitrogen in the seeds of Hiproly compared to the normal seeds. Qualitative differences were noted for the water-soluble extracts as revealed by polyacrylamide-gel electrophoresis. Tallberg (13), studying unripe seeds of Risø No. 1508, found qualitative differences in water-soluble proteins in polyacrylamide electrophoresis compared to Bomi barley. Pomeranz *et al.* (14) noted that the amount of lysine and aspartic acid per kernel was identical in mature kernels of Hiproly and CI

[1]Research carried out under contract No. 138-74-1 BIO-DK with the European Atomic Energy Community.

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4362, and suggested that differences in concentrations of amino acids/g protein in the two barleys resulted from differences in the composition of the proteins in the various kernel tissues.

This paper describes results of a study on the developmental sequence of reserve protein deposition in the endosperm of Bomi barley and mutant No. 1508 to provide additional information on the possibilities of changing the amino acid composition of the barley kernel and improving its content of nutritionally desirable amino acids and proteins.

MATERIALS AND METHODS

Plant Material

Bomi barley (*Hordeum vulgare* L. cv. Bomi) and its high-lysine mutant, Risϕ No. 1508, were field-grown. Spikes were harvested 8, 13, 20, 28, and 42 days after fertilization, frozen in liquid nitrogen, and stored at −18°C until needed for analysis.

Extractions

The extractions were done on endosperms isolated as follows: lemma, palea, and embryo were removed with tweezers. The endosperm was then squeezed out of the green pericarp, except in the case of the 8- and 42-day-old endosperms, where the last step was not feasible.

Free amino acids were extracted from ten endosperms by boiling ethanol 80% (v/v). The extract was reduced in volume at <40°C, redissolved in 0.01*N* HCl, and applied to a Dowex-50 W, H^+, (200–400 mesh) column. Organic acids and sugars were eluted with 1*N* HCl and the amino acids eluted from the column with 2*N* NH_3 (15). The composition of the fraction was determined on an automatic amino acid analyzer without prior hydrolysis.

Protein was extracted successively from 20 endosperms with 3 × 5 ml H_2O at 4°C for 30 min (albumins); 3 × 5 ml 0.5*M* NaCl at 4°C for 30 min (globulins); 3 × 5 ml 55% (v/v) isopropanol at 20°C for 30, 60, and 120 min (hordeins); and 3 × 5 ml 0.2*N* NaOH at 20°C for 30, 60, and 120 min (glutelins). After each extraction step, the extracts were centrifuged at 20,000 × *g* and the supernatants combined to give the appropriate fractions (16).

Nitrogen Determinations

The nitrogen contents of the digested fractions were determined in duplicate by Nesslerization (17), and protein was calculated as N × 6.25.

Amino Acid Analysis

The amino acid composition of the hydrolyzed fractions was determined by automatic ion-exchange chromatography on a Beckmann 120C. Hydrolysis was carried out in vacuum with 6*N* HCl at 110°C for 18 hr.

Electrophoretic Analysis

Samples for electrophoresis were prepared by dialysis of the extracts against 0.06*M* tris-borate buffer, pH 8.9, containing 1% (w/v) sodium dodecyl sulfate (SDS) and 2% (v/v) β-mercaptoethanol at 20°C for 18 hr. Prior to dialysis, SDS was added to the isopropanol extracts. Glutelin for electrophoresis was extracted

with the SDS, β-mercaptoethanol containing buffer at 40°C for 2 hr prior to dialysis. Electrophoresis was performed in 7.5% polyacrylamide-gel tubes containing 0.1% SDS in 0.06*M* tris-borate buffer at pH 8.9. The gels were pre-electrophoresed at constant current, 2.5 mA/gel for 1.5 hr. Approximately 100 μg protein was mixed with sucrose and layered on top of the gels. Electrophoresis was carried out at constant current, 2.5 mA/gel for 80 min toward the anode. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R250 in 6% trichloroacetic acid. A mixture of acetic acid:methanol:water (20:120:80 v/v/v) was used for destaining. Molecular weight (mol wt) calibration of the gel system was performed in a seprate gel in each electrophoretic run with bovine serum albumin (mol wt 67,000), ovalbumin (mol wt 45,000), trypsin (mol wt 24,000), trypsin inhibitor (soybean) (mol wt 21,500), and cytochrome C (mol wt 13,000) (Sigma Chem. Co., Inc.) treated as the barley proteins. The gels were scanned at 620 nm with a Joyce Loeb Chromoscan 200.

RESULTS

In Bomi barley and in the high-lysine mutant endosperms, the dry weight increased rapidly during the first 20 days after fertilization (Fig. 1). Thereafter, only smaller increases in dry weight were noted. From the second week after fertilization, the dry weight of the mutant endosperm amounted to only 90% of that of Bomi barley.

As can be seen from Fig. 2, more than 50% of the albumins were already present 8 days after fertilization. Synthesis of these proteins reached saturation between 13 and 20 days after fertilization. Very small amounts of the globulins, hordeins, and glutelins were formed during the first 13 days after fertilization. In Bomi barley (Fig. 2A), hordeins and glutelins were thereafter synthesized at increasing rates. Synthesis reached saturation from 28 to 42 days after fertilization. The highest rate of accumulation of these proteins occurred after

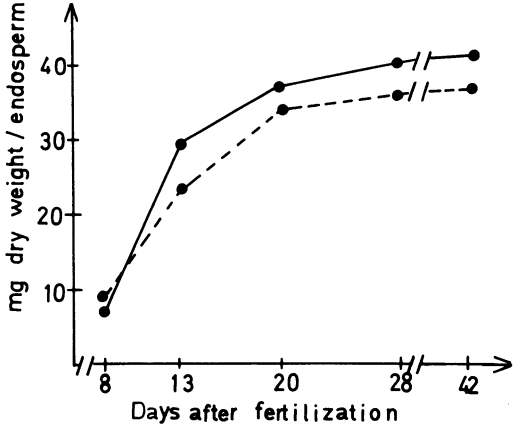

Fig. 1. Increase of endosperm dry weight during kernel development of Bomi barley (_____) and mutant No. 1508 (- - - -). The kernels were physiologically mature at 28 days and combine-ripe at 42 days after fertilization.

the major synthesis period for the albumins was completed. Hordeins and glutelins were the major reserve proteins comprising 30% each of the endosperm proteins, whereas the globulins comprised 10% of the endosperm proteins of Bomi barley at 42 days after fertilization.

From the beginning, mutant No. 1508 showed a low level of hordein production (Fig. 2B). In contrast to Bomi barley, this production takes place at a constant rate without further increase 13 days after fertilization. The impaired hordein synthesis in the mutant endosperm results in only 8% of the total endosperm protein being in this fraction at 42 days after fertilization, whereas the amount of protein present in the globulin and glutelin fractions of the mutant is the same as in Bomi barley. The increased amount of nitrogen in the albumin fraction of the mutant endosperm, as compared to Bomi barley (Fig. 2), is mostly due to an increase in the amount of free amino acids (Table I). The free amino acid pool in Bomi barley is constant throughout development, comprising about 15 μg N/endosperm. In contrast, the amount of free amino acids in the mutant endosperm is three times as high at all developmental stages. The amount of free glutamic acid in Bomi barley increases and the amount of glycine decreases. The other free amino acids do not seem to change appreciably in amount during endosperm development. In the mutant, the relative amount of glutamic acid is reduced, compared to Bomi barley, but increases toward advanced stages of endosperm development. Proline, aspartic acid, threonine, and serine make up a larger part of the free amino acids in the mutant endosperm than in Bomi barley. In contrast, the amount of alanine is drastically reduced in the mutant at a later stage of endosperm development.

Amino Acid Composition of Albumins and Globulins

The amino acid composition of the albumins and globulins at 28 days after fertilization is given in Table II. The amino acid composition of the albumins is

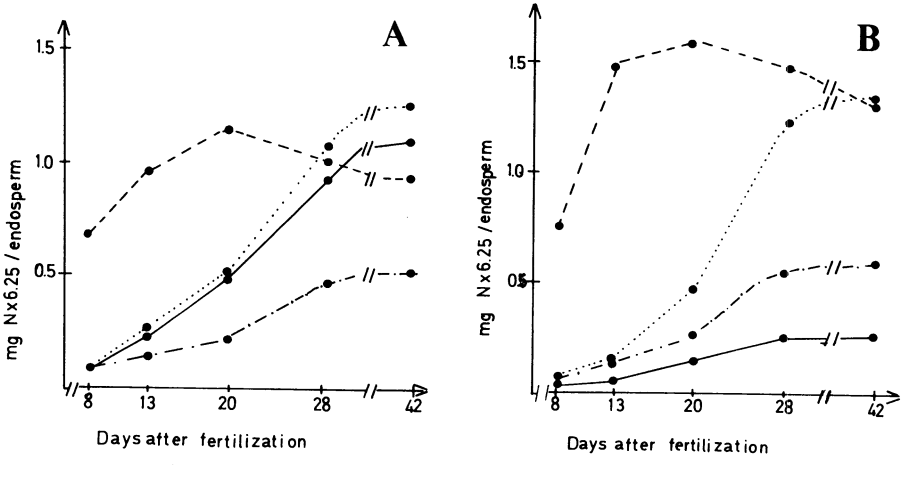

Fig. 2. Changes in the endosperm protein fractions during kernel development of Bomi barley (A) and mutant No. 1508 (B). Albumins (including free amino acids), - - - - -; glubulins, -.-.-.; hordeins, _____; and glutelins, (.....).

TABLE I
Free Amino Acids[a] in Endosperms during Development of Bomi Barley and Mutant 1508

Amino Acid	Bomi			1508		
	13 DAF[b]	20 DAF	28 DAF	13 DAF	20 DAF	28 DAF
Lysine	4	2	2	2	2	2
Histidine	1	1	...[c]	...[c]	...[c]	1
Arginine	1	1	1	1	3	3
Aspartic acid	3	5	5	2	10	10
Threonine	12	8	9	14	17	15
Serine	13	10	12	15	19	15
Glutamic acid	17	31	27	5	8	20
Proline	6	7	5	6	13	10
Glycine	11	3	0	9	1	2
Alanine	22	24	24	35	16	8
Cysteine	0	0	0	1	1	...[c]
Valine	4	3	5	3	5	7
Methionine	0	0	0	0	0	0
Isoleucine	2	1	3	2	1	2
Leucine	2	2	3	2	2	3
Tyrosine	1	1	2	1	1	1
Phenylalanine	1	1	2	1	1	2
μg N/endosperm	17	17	12	58	50	55

[a]Calculated as mole per cent of all amino acid residues.
[b]DAF = days after fertilization.
[c]Trace.

TABLE II
Amino Acid Composition (mole per cent) of Albumin and Globulin Protein Fractions from Bomi Barley and its High-Lysine Mutant 1508, 28 Days after Fertilization

Amino Acid	Albumin		Globulin	
	Bomi	1508	Bomi	1508
Lysine	5.1	6.1	5.3	5.8
Histidine	1.9	1.9	1.7	2.2
Arginine	3.8	4.9	5.5	6.4
Aspartic acid	10.4	9.6	8.1	7.9
Threonine	4.6	5.0	4.4	4.5
Serine	5.4	5.9	5.4	4.7
Glutamic acid	14.5	11.5	15.5	12.7
Proline	7.3	6.0	9.3	6.7
Glycine	8.9	9.7	9.6	10.8
Alanine	10.6	9.8	7.9	8.4
Cysteine	0.5	0.5	0	0
Valine	7.2	7.4	7.6	5.3
Methionine	1.9	2.1	1.8	1.5
Isoleucine	4.1	4.6	3.8	3.5
Leucine	7.9	9.3	7.9	13.6
Tyrosine	2.4	2.6	2.8	2.7
Phenylalanine	3.7	3.2	3.6	3.5

nearly constant throughout endosperm development in both genotypes and does not differ significantly at earlier stages from the composition of the fraction at 28 days after fertilization. On the other hand, the amount of glutamic acid and proline increased slightly in the globulin fraction during endosperm development. The albumins and globulins of the mutant endosperm appear to be lower in glutamic acid and proline as compared to Bomi.

Amino Acid Composition of the Hordeins

The hordeins of Bomi barley endosperm are rich in glutamic acid and proline, the two amino acids accounting for nearly 60% of this fraction at 28 days after fertilization (Table III). The increased formation of these proteins at 13 days after fertilization is characterized by a sudden increase in glutamic acid and proline with a corresponding decrease in other amino acids, especially lysine, aspartic acid, serine, glycine, and alanine. Thereafter, the amino acid composition does not seem to change appreciably. The same overall pattern is evident in the hordeins of the mutant endosperm. Glutamic acid and proline comprise only 50% of the proteins at an advanced stage of endosperm development, and the amounts of lysine, aspartic acid, and glycine are correspondingly more abundant than in Bomi barley.

Amino Acid Composition of the Glutelins

The amino acid composition of the glutelins is different from that of the albumins, globulins, and hordeins (Table III). As in the hordein fraction, there is an increase in glutamic acid and proline during the early stages of glutelin formation; in contrast to hordein, however, the glutelin fraction reveals successive changes in the amino acid composition as endosperm development progresses. Glutamic acid and proline (in the proteins) at 28 days after fertilization total 40% in Bomi and 25% in the mutant. Consequently, relatively more lysine and aspartic acid are present in this fraction in the mutant endosperm compared to Bomi barley.

Electrophoretic Analysis

All endosperm proteins entered the gels in SDS-polyacrylamide gel electrophoresis except for the glutelin fraction, in which some protein remained on top of the gels. A modified extraction procedure was used to overcome this situation (see **Materials and Methods**). Upon reduction, all endosperm protein fractions contained a component giving a diffuse band with an apparent mol wt of 20,000 (Fig. 3). This component was used as reference, and the other bands of the different protein fractions were compared to the intensity of this band.

The albumin fraction (Fig. 3A) contains major bands with mol wt of 95,000 and 70,000 in both genotypes. The bands in the mol-wt range of 95,000–58,000 are higher in the mutant. During endosperm development, there were only minor modifications of electrophoretic patterns in the two genotypes; this is consistent with the stability in the amino acid composition of the above fraction.

At 13 days after fertilization, the globulins consist primarily of low-molecular-weight components (Fig. 3B). At 20 to 28 days after fertilization, higher molecular-weight components appear and contribute increasingly to the protein fraction, which is consistent with the gradual changes in the protein deposition and amino acid composition of the fraction during endosperm development. No

TABLE III
Amino Acid Composition (mole per cent) of Hordein and Glutelin Endosperm Protein Fractions from Bomi Barley and its High-Lysine Mutant No. 1508 at Different Days after Fertilization (DAF)

Amino Acid	Hordeins						Glutelins					
	Bomi			1508			Bomi			1508		
	8 DAF	13 DAF	28 DAF	8 DAF	13 DAF	28 DAF	8 DAF	13 DAF	28 DAF	8 DAF	13 DAF	28 DAF
Lysine	3.5	0.6	0.5	3.3	2.2	1.0	6.5	4.4	2.2	6.5	4.4	4.4
Histidine	1.5	0.7	1.0	1.5	0.7	0.8	2.3	2.0	1.9	2.0	1.9	2.4
Arginine	2.0	1.9	2.0	4.1	1.8	2.1	4.8	3.9	3.2	4.9	4.1	4.8
Aspartic acid	8.0	2.4	1.6	9.4	6.9	3.4	7.2	5.8	4.2	9.5	9.1	7.5
Threonine	5.4	2.5	2.0	4.6	4.9	2.9	4.2	4.3	3.8	5.0	5.2	4.9
Serine	8.4	4.9	4.2	8.4	6.7	5.1	6.6	6.3	5.7	6.4	7.2	6.2
Glutamic acid	12.9	34.1	34.7	12.9	23.9	31.2	15.0	18.7	25.9	10.9	14.7	17.2
Proline	7.9	20.9	23.0	8.0	11.6	19.0	8.2	11.1	14.1	6.2	7.4	8.3
Glycine	8.9	3.6	2.5	13.5	8.3	5.4	9.0	9.3	6.3	10.3	11.4	9.4
Alanine	8.0	3.1	2.4	10.1	7.2	3.8	7.6	6.7	5.1	9.4	7.3	7.3
Cysteine	0.0	0.3	1.6	0.0	0.0	0.2	0.0	0.0	0.5	0.0	0.0	0.1
Valine	7.9	5.0	4.6	9.6	6.3	4.9	6.5	6.8	6.5	7.1	7.3	6.8
Methionine	3.3	1.0	0.9	0.6	1.6	1.0	2.0	1.8	1.3	1.6	0.6	1.7
Isoleucine	6.1	3.7	3.8	5.8	3.3	3.4	5.2	4.8	4.2	4.4	3.7	4.3
Leucine	8.1	7.4	6.9	0.8	9.1	7.8	8.3	7.9	8.2	9.3	8.3	8.1
Tyrosine	3.6	2.3	2.4	2.9	1.4	2.3	2.6	2.7	2.7	2.5	2.5	2.8
Phenylalanine	4.5	5.6	5.9	4.6	4.5	5.1	4.1	3.5	4.3	4.1	3.9	3.8

qualitative differences between the electrophoretic patterns of the mutant and of the Bomi globulins are noticeable, but the 68,000 mol wt band of the mutant globulins appears to be more intensely stained than the band with the same electrophoretic mobility in the Bomi barley globulins.

Bomi barley hordeins are composed of 6 bands in the mol wt range of 80,000–20,000 (*i.e.*, 20,000, 45,000, 55,000, 60,000, 68,000, and 80,000 mol wt bands) and these are present from the earliest stage of hordein formation (13 days

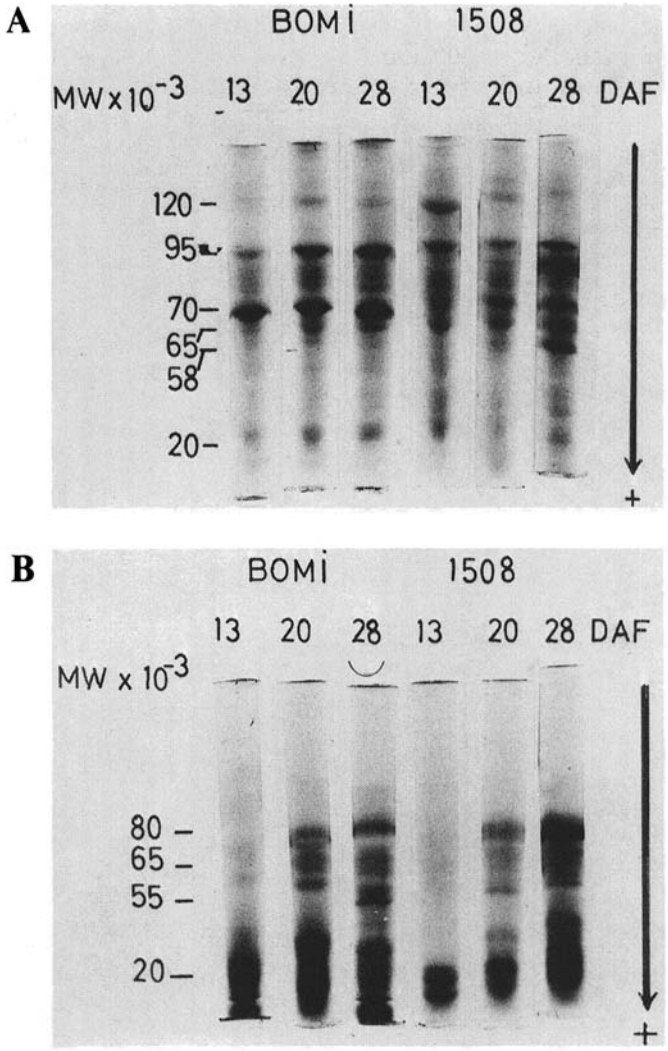

Fig. 3. SDS-polyacrylamide gel electrophoretic analysis of the reduced endosperm proteins from Bomi barley and mutant No. 1508 at different stages of endosperm development. A = albumins, B = globulins.

after fertilization). The intensity of the components of the protein fraction with mol wt of 80,000–45,000 increases relative to the 20,000 mol wt during endosperm development (Fig. 4A). These components account for the rapid increase in the concentration of glutamic acid and proline in the protein fraction, whereas the 20,000-mol wt component, which is the only band present at 8 days after fertilization (data not shown), contributes most of the amount of lysine in the protein fraction. The banding pattern of the mutant endosperm hordein is quite different. The band with an apparent mol wt of 20,000 remains a significant component throughout all endosperm developmental stages. The bands with mol wt of 80,000, 68,000, and 45,000 (labeled 50,000 in Fig. 4A) are present, but the bands with mol wt 60,000 and 55,000 are not synthesized.

Five major bands are apparent in Bomi barley glutelins in the mol wt range of

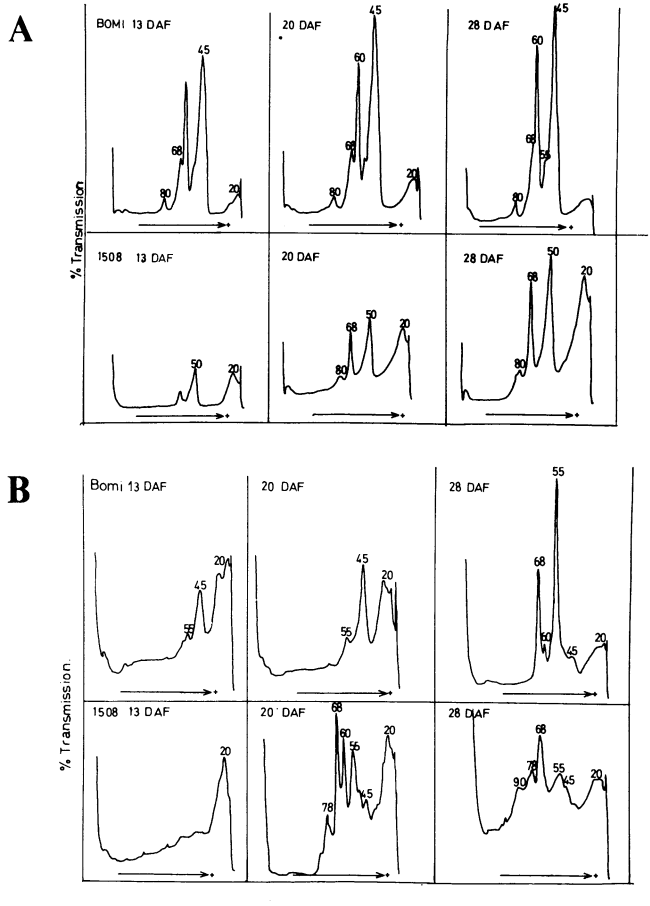

Fig. 4. Densitometric tracings of the reduced hordein and glutelin fractions (after SDS polyacrylamide gel electrophoresis) at different stages after fertilization. A = hordeins, B = glutelins. Upper row, Bomi barley; lower row, mutant No. 1508. Direction of electrophoresis, left to right.

68,000–20,000 (*i.e.*, 20,000, 45,000, 55,000, 60,000, and 68,000, Fig. 4B). The major bands at an advanced stage of endosperm development are the 68,000 and 55,000 mol wt components. At an early stage of endosperm development, only the bands with mol wt of 45,000 and 20,000 are present, and the components with apparent mol wt of 68,000 and 60,000 appear between 20 and 28 days after fertilization. The mutant glutelins are composed of low-molecular-weight bands at early stages of endosperm development; as endosperm development progresses, a series of six bands in the mol wt range of 68,000–45,000 is formed and additional bands compared to Bomi barley with mol wt of 78,000 and 90,000 appear at 28 days after fertilization. The different band pattern in the glutelin fraction of the mutant endosperm has its counterpart in the altered amino acid composition of this fraction compared to Bomi barley.

DISCUSSION

As noted by Munck (12) the solubility of the barley endosperm proteins in the Osborne extraction procedure is to some extent dependent on the ripeness of the kernels. In spite of this, the foregoing results allow one to recognize the pattern of synthesis of these four major classes of proteins during endosperm development, and to define the differences between the high-lysine mutant, Risφ No. 1508, and its mother variety Bomi barley.

The intensive synthesis of hordeins, which can be followed in Bomi barley endosperm, is severely impaired in the mutant throughout endosperm development, leading to the low amount of hordeins present at maturity, as observed by Ingversen *et al.* (3). The mutant synthesizes four of the six components present in Bomi barley hordeins, whereas two major components are missing or present in trace amounts only. The lower molecular-weight components contribute more to the hordein fraction in the mutant than they do in Bomi barley hordeins. These components with a mol wt of 20,000 are known to have relatively high concentrations of lysine compared to the other hordein components (18). As the Bomi barley hordeins extracted at 8 days after fertilization almost exclusively consist of these components (gels not shown), the lysine content at this stage of endosperm development is relatively high compared to the hordein fraction at more advanced stages of endosperm development (Table III). Due to the low amount of protein in the hordein fraction in the mutant, the absolute amount of lysine per endosperm in this fraction is 2.5 times less than in Bomi barley hordein at advanced stages of endosperm development. The complete inhibition of two components and the impairment of the synthesis of the other hordein components in the mutant endosperm indicate that the small amount of hordeins at maturity is not due to a later onset of hordein synthesis, as has been reported for *opaque*-2 maize zein (19).

In Bomi barley, the changes in amino acid composition of the glutelins (Table III) and their electrophoretic patterns (Fig. 4B) suggest that this fraction shares components with the hordeins (Fig. 4A). This is verified by coelectrophoresis of the fractions (unpublished data), although no explanation of the solubility differences of the proteins can be given. Landry *et al.* (20) suggested that the barley glutelins are composed of two groups of proteins, one with amino acid composition similar to the albumins/globulins and one with amino acid

composition similar to the hordeins. A preferential inhibition of hordein components in the glutelin fraction could explain the changed protein banding pattern and the altered amino acid composition of the glutelins in the mutant endosperm. The presence of similar polypeptides in the proteins of different solubility classes when reduced and unfolded with SDS emphasizes the limitations of the classical protein extraction procedure to elucidate protein formation in the barley endosperm.

The differences in the amounts of salt-soluble nitrogen in the mutant compared to Bomi barley at maturity reported by Ingversen *et al.* (3) account for the increased amount of free amino acids (Table I), since great differences in the amino acid composition and electrophoretic patterns are not observed in the albumins or the globulins. Among the changes in composition of free amino acids in the mutant endosperm, the increase in aspartic acid and the strong decrease in glutamic acid may be especially significant in view of the central position of these amino acids in the transamination.

The increased amount of free amino acids could have resulted from the inhibition of hordein synthesis. An increasing accumulation of free amino acids during mutant endosperm development would be expected in this case; however, this does not occur. The relationship between the large amounts of free amino acids and the inhibition of hordein synthesis will have to be explored in further studies.

It is concluded that the increased amount of lysine in the mutant endosperm is due to a severe impairment of hordein synthesis, a reduction of lysine-poor components of the glutelin fraction with a compensating increase in lysine-rich components in this fraction, as well as an elevated amount of free lysine.

Acknowledgments

I thank J. Ingversen and D. von Wettstein for advice and useful discussions. The technical assistance of S. Djurdjevic is acknowledged.

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[Received October 17, 1975. Accepted Februàry 23, 1976]