Amino Acid Composition of Proteins Isolated from Normal, Opaque-2, and Floury-2 Corn Endosperms by a Modified Osborne Procedure

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The classical Osborne–Mendel extraction procedure for corn endosperm protein was modified to extract additional protein by a solvent containing 55% 2-propanol and 0.6% 2-mercaptoethanol. The new fraction is designated zein-2, since it has an amino acid composition somewhat similar to zein, but with higher levels of glycine, methionine, histidine, and proline, and lower levels of aspartic acid, leucine, and isoleucine. Zein-2 was previously recovered as a part of the glutelin fraction. Zein-1 and zein-2, as well as glutelin, were heterogeneous by polyacrylamide gel electrophoresis. However, these three protein fractions had characteristic amino acid compositions which were constant for normal, opaque-2, and floury-2 endosperms. The two mutations which increase the lysine content of the endosperm act by changing the proportions of proteins which contain different levels of lysine.

Since the discovery of the high lysine content in the endosperm of the opaque-2 corn (Mertz *et al.*, 1964), considerable attention has been given to the effect of this gene on the synthesis of storage proteins. The major effect is a reduction in the amount of zein produced (Mertz *et al.*, 1964; Mosse, 1966; Mosse *et al.*, 1966; Paulis *et al.*, 1969; Sodek and Wilson, 1970). Since zein is essentially devoid of lysine, a reduction in zein results in an increased proportion of lysine, especially if the other proteins are increased. There does not seem to be any change in the amino acid composition of the nonzein proteins (Mosse *et al.*, 1966), but the evidence is somewhat conflicting (Mertz *et al.*, 1964). The other major storage protein is glutelin, but extraction procedures based on the work of Osborne and Mendel (1914) leave variable amounts of zein in the glutelin fraction (Dalby, 1966; Dimler, 1966; Paulis *et al.*, 1969). Zein appears to be cross-linked to glutelin through disulfide bonds (Paulis *et al.*, 1969). Certain of the globulins may also be associated with the glutelin fraction (Boundy *et al.*, 1967; Landry and Moureaux, 1970). Another mutant gene, floury-2, also changes the lysine content of corn by changing the proportions of the proteins (Jimenez, 1968; Mosse, 1966; Nelson *et al.*, 1965).

Moureaux and Landry (1968) used several solvent systems in modifications of the Osborne–Mendel procedure to obtain better protein fractions without the use of reagents which might degrade the proteins. After zein was extracted from whole seeds, the residue was further extracted with alcohol which contained 2-mercaptoethanol to reduce the disulfide links between proteins. They obtained an alcohol-soluble fraction which contained about 10% of the total nitrogen of the seed and which had an amino acid composition similar to zein, but richer in methionine. Since the formation of disulfide bonds has been implicated as a cause for cross-contamination of protein fractions, we tried this modification of the classical Osborne extraction and did obtain better separation of zein and glutelin. Landry and Moureaux (1970) issued a more detailed report, including amino acid composition data, after we had completed our study.

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In this paper we are presenting relative protein concentrations and amino acid compositions of the proteins extracted from normal, opaque-2, and floury-2 endosperms by a modification of a Moureaux and Landry (1968) solvent system. The results demonstrate that the two mutations have little effect on the amino acid composition of the various protein fractions, and that 2-mercaptoethanol increases the amount of extractable zein. The amino acid composition of the glutelin fraction is changed by the modified extraction procedures, since glutelin is much less contaminated with zein.

EXPERIMENTAL

Plant Material. Corn (*Zea mays* L.) was grown in the field during the summers of 1968 and 1969. Lines included homozygous normal, opaque-2, and floury-2 versions of the inbred R802; the F2 seed of the normal version of the hybrid WF9 × M14, and the F2 seed of the opaque-2 version of the hybrid R802 × R75. Normal and opaque-2 kernels (1:1 ratio) obtained from a segregating ear of R802 pollinated by a homozygous opaque-2 plant provided the material for Tables III through VI. The ears were harvested 50 days after pollination and frozen. The kernels were dehulled, the germ was removed, and the endosperm was lyophilized. The endosperm was ground to a powder in a Spex mill.

Fractionation of Proteins. The proteins were successively extracted by a modified Osborne method using mercaptoethanol as suggested by Moureaux and Landry (1968). However, we did not further subdivide the glutelin fraction. The extraction scheme is outlined in Table I. The protein in each fraction was precipitated by the addition of 25% trichloroacetic acid to a final concentration of 5%. The alcohol extracts were first diluted with one volume of water. The precipitates were collected by filtration through glass fiber disks (Whatman GF/C), which were then washed with 5% TCA and dried under a lamp. The albumin, globulin, and glutelin precipitates were left overnight at 4° C before collection and the zein-2 was left for about 3 hr; but the zein-1 was collected after 1 min to avoid the sticking of clumps to the wall of the test tube. This procedure was designed for the assay of large numbers of samples for ^{14}C analysis (Sodek and Wilson, 1970). The trichloroacetic acid-soluble fraction of the water extract contained the free amino acids. This fraction was shaken four times with 1 vol of

Table I. Sequential Extraction of Proteins from Corn Endosperm

Fraction		Solvent	Extractions[a] min
Free amino acids	1.	Water, 0° C	30, 30, 30, and wash
Albumins Globulins	2.	Water,[b] 0° C 0.5 *M* NaCl, 0° C	30, 30, 30, and wash
Zein-1	3.	55% 2-propanol, 20° C	3, 120, 60, and wash
Zein-2	4.	55% 2-propanol, 0.6% mercaptoethanol, 20° C	30, 60, 30, and wash
Glutelin	5.	0.2% NaOH, 20° C	30, 120, 60, and wash
Residue		Insoluble	

[a] Samples of 100 mg of finely-ground lyophilized endosperm were extracted by stirring with 1 ml of solvent in conical centrifuge tubes. At the end of each fractionation step the residue was washed with 1 ml of water (fractions 1, 2, 5) or with 0.5 ml 55% 2-propanol (fractions 3 and 4) and the washings were combined with the appropriate extracts. The supernatant solutions obtained after centrifugation at 1000 × *g* for 10 min were combined. [b] The albumins were separated from the free amino acids by precipitation with 5% trichloroacetic acid.

ether to remove the trichloroacetic acid and the aqueous phase was concentrated on a rotary evaporator for further study.

Nitrogen was determined by a micro-Kjeldahl method.

Special Fractionation of Glutelin. A glutelin fraction was obtained much like the classical Osborne fraction by omitting step 4 (Table I). 2-Propanol was added to the NaOH extract to make a 60% solution, the pH was carefully lowered to 6 with 1 *N* HCl, and the solution was left at 4° C overnight. A white flocculent precipitate was collected by centrifugation. The supernatant was diluted with 1 vol of 5% trichloroacetic acid and 25% trichloroacetic acid was added to make a final concentration of 5%. After several hours at 0° C, the precipitate was collected by filtration through a glass fiber disk.

Amino Acid Analysis. The proteins were hydrolyzed in evacuated sealed tubes with 6 *N* HCl for 24 hr at 110° C. Amino acids were determined according to Moore *et al.* (1958) on an automatic amino acid analyzer. Amides in the free amino acid fraction were determined by the differences in aspartic acid and glutamic acid contents before and after hydrolysis for 3 hr in 1 *N* HCl. Tryptophan was not determined, and cystine was sometimes destroyed during hydrolysis. Methionine may be oxidized to several different products during hydrolysis, but none of the products were noted on the chromatograms. The high levels of methionine and cystine in some samples were confirmed by converting these amino acids to methionine sulfone and cysteic acid by performic acid oxidation (Moore, 1963). The results were similar to those obtained by the regular evacuated tube method, and are not reported separately.

Gel Electrophoresis. Polyacrylamide disk gel electrophoresis was carried out using two procedures modified to meet the special solubility problems encountered with zein. A pH 8.9 tris-HCl gel with tris-glycine electrode buffer, pH 8.3, was prepared according to Davis (1964), but with 6 *M* urea in the gel. A pH 2.9 tris-citric acid gel with a glycine–citric acid electrode buffer was prepared according to Jordan and Raymond (1969), also with 6 *M* urea in the gel. The gels were prepared in 5 × 75 mm glass tubes. Both gels contained 5% polyacrylamide. After electrophoresis at 4 mA/tube for 1 or 2 hr, the gels were stained with Amido black.

Zein extracts in 60% 2-propanol were prepared for electrophoresis by adding solid urea, then removing the 2-propanol on a rotary evaporator. Glutelins were precipitated by adjusting the alkaline extract to pH 5.9 and then centrifuging. The precipitate was dissolved in 8 *M* urea. The disulfide bridges in the proteins were reduced by adding 0.3% 2-mercaptoethanol to the samples before electrophoresis. Without prior reduction, zein does not enter the gel. The samples, with sucrose added to increase the density, were layered over the top of the gel. The electrode buffer in contact with the samples contained 6 *M* urea.

RESULTS

Protein Composition. The distribution of nitrogen among the protein fractions is given in Table II. Total nitrogen was lower in the opaque-2 version of R802 than in the normal counterpart, but the normal and opaque-2 hybrids had the same nitrogen concentration. The opaque-2 gene characteristically depressed the level of zein in both the inbred and hybrid plants, but the effect was much greater in the inbred, where zein-1 was essentially missing. The opaque-2 gene had much less effect on the amount of zein-2. All of the nonzein fractions increased in absolute amounts and in percentage of total nitrogen in the opaque-2 endosperms, though in the inbred the amount of glutelin was only slightly increased. The level of free amino acid was much higher in the opaque-2 endosperm than in the normal or floury-2 endosperms. The floury-2 gene increased the glutelin fraction, but had little effect on zein.

Endosperm Amino Acid Composition. The amino acid compositions of the inbred and hybrid normal, opaque-2,

Table II. Distribution of Nitrogen in Endosperm Protein Fractions of Normal, Opaque-2, and Floury-2 Inbreds and Hybrids

	R802 normal		R802 opaque-2		WF9 × M14 normal		R802 × R75 opaque-2		R802 floury-2	
	mg N/g	%	mg N/g	%	mg N/g	%	mg N/g	%	mg N/g	%
Free amino acid	0.80	4.4	2.50	19.7	0.47	2.9	1.45	9.1	0.44	2.2
Albumin	0.16	0.9	0.42	3.3	0.28	1.7	0.58	3.6	0.27	1.3
Globulin	0.27	1.5	0.60	4.7	0.52	3.2	0.83	5.2	0.54	2.7
Zein-1	6.66	36.9	0.37	2.9	6.75	41.9	2.84	17.8	7.47	36.6
Zein-2	3.34	18.5	1.73	13.6	3.02	18.8	2.21	13.9	3.12	15.3
Glutelin	4.13	22.9	4.72	37.2	2.95	18.3	5.63	35.3	5.60	27.4
Total	15.36	85.1	10.34	81.4	13.99	86.8	13.54	84.9	17.44	85.5
Whole endosperm	18.05		12.70		16.10		15.95		20.4	
Endosperm dry wt	109 mg		116.5 mg		257 mg		168 mg		119 mg	

Table III. Total Amino Acid Composition of Endosperms from Normal, Opaque-2, and Floury-2 Inbreds and Hybrids

	μmol/100 μmol				
	R802 normal	R802 opaque-2	WF9 × M14 normal	R802 × R75 opaque-2	R802 floury-2
Lysine	1.7	4.1	1.5	3.2	2.5
Histidine	1.6	2.2	2.1	2.4	1.6
Arginine	2.1	3.6	2.1	3.2	2.5
Aspartic acid	6.1	11.2	5.5	9.4	6.7
Threonine	3.2	4.0	3.6	3.7	3.6
Serine	5.4	5.3	5.6	5.3	5.2
Glutamic acid	17.9	17.7	18.3	16.4	17.0
Proline	11.9	10.3	13.6	11.9	10.9
Glycine	4.6	7.8	5.5	6.7	5.3
Alanine	14.1	8.3	11.8	9.7	11.4
Half-cystine	1.1	1.1	1.1	1.1	1.1
Valine	3.9	5.7	5.3	5.5	5.3
Methionine	2.0	1.9	1.7	1.6	2.4
Isoleucine	3.6	3.4	3.3	3.5	3.9
Leucine	14.0	7.8	13.4	10.3	13.9
Tyrosine	2.9	2.5	2.9	2.7	3.0
Phenylalanine	3.8	3.1	3.6	3.5	3.9

and floury-2 endosperms are given in Table III. As expected, the opaque versions contained higher proportions of lysine, histidine, arginine, aspartic acid, and glycine, and lower proportions of glutamic acid, proline, and leucine. These differences are directly related to the lower zein content, except for aspartic acid, a major component of the high free amino acid fraction. The floury-2 composition is intermediate between the normals and the opaques.

Amino Acid Composition of the Free Amino Acids, Albumins, and Globulins. Only a single pair of samples was analyzed for these fractions (Table IV), for the emphasis in this paper is on the major storage proteins. The free amino acid fractions contained relative amounts of amino acids which differed greatly from the total endosperm or any of the protein fractions. There were high levels of aspartic acid, glutamic acid, proline, and the two amides, which accounted for most of the nitrogen of this fraction. In contrast, leucine, which is very abundant in the proteins, had one of the smallest pools of free amino acid. The opaque-2 endosperm contains relatively higher percentages of free glutamic acid and glutamine, and lower proline than the normal, and there is also more free lysine.

The amino acid compositions of the albumin and globulin fractions were very similar in both normal and opaque-2 endosperms (Table IV). Paulis and Wall (1969) reported some differences between albumins and globulins, but their values were not greatly different from the ones reported here.

Amino Acid Composition of Zein-1 and Zein-2. The amino acid composition of zein-1 (Table V) from four types of endosperm was essentially identical. Zein-1 contained the characteristic high contents of glutamic acid, proline, and leucine, and low contents of lysine and other basic amino acids. Zein-2 (Table V) has a general composition similar to zein-1, and can be clearly distinguished from the nonzein fractions on this basis. However, there are several differences between the two zeins. The glycine content was doubled and the methionine content increased up to 5.6% in zein-2. The latter values were confirmed by oxidizing methionine to methionine sulfone before analysis. Histidine and proline were higher in zein-2, especially in the two hybrid samples, while aspartic acid, leucine, and isoleucine were lower than in zein-1.

Amino Acid Composition of Glutelin and Residue Proteins. The glutelin fractions of normal, opaque-2, and floury-2 endosperms were mostly similar in overall amino acid composition (Table VI). They were intermediate between the albumins and globulins and the zeins for most amino acids, but more like the former proteins. The lysine content of the inbred opaque-2 glutelin was not different from the normal, while the opaque-2 hybrid and floury-2 glutelins contained 5.5% lysine. Zein-2 will be found in either the glutelin fraction or the residue when the regular Osborne extraction procedure is followed. The amino acid composition of an Osborne glutelin fraction is shown in Table VII. It differs from the glutelins in Table VI, but for almost all of the amino acids the values are intermediate between those for zein-2 (Table V) and those for glutelin, *i.e.*, lysine, aspartic acid, glutamic acid, methionine, and leucine. The Osborne glutelin was divided into a subfraction insoluble in

Table IV. Amino Acid Composition of the Free Amino Acid, Albumin, and Globulin Fractions of Normal and Opaque-2 R802 Endosperm

	μmol/100 μmol					
	Free amino acid		Albumin		Globulin	
	Normal	Opaque-2	Normal	Opaque-2	Normal	Opaque-2
Lysine	2.5	4.2	5.4	5.6	5.0	5.2
Histidine	0.6	0.8	1.9	1.8	2.0	1.9
Arginine	1.1	1.5	5.3	4.9	5.5	6.0
Aspartic acid	24.8	25.9	8.2	9.2	8.6	9.3
Threonine	1.2	1.0	5.0	5.4	4.9	4.7
Serine	4.6	4.0	5.6	5.6	5.6	5.3
Glutamic acid	17.4	21.3	12.3	10.6	11.6	10.9
Proline	19.6	8.6	6.9	6.6	7.4	6.9
Glycine	4.4	1.9	10.1	10.5	11.3	12.2
Alanine	5.9	2.2	11.0	10.4	10.4	10.7
Half-cystine		0.3	0.2	1.4	1.2	0.3
Valine	1.1	1.4	7.1	7.6	7.0	7.1
Methionine			1.3	1.3	1.2	1.3
Isoleucine	0.5	0.6	4.4	4.7	4.1	4.0
Leucine	0.6	0.8	9.3	8.6	8.1	7.6
Tyrosine	1.7	1.5	2.5	2.6	2.6	3.0
Phenylalanine	1.0	0.9	3.3	3.3	3.6	3.7
Asparagine	7.9	7.2				
Glutamine	4.9	15.9				

Table V. Amino Acid Composition of Zeins from Endosperms of Normal, Opaque-2, and Floury-2 Inbreds and Hybrids

	μmol/100 μmol								
	Zein-1				Zein-2				
	R802 normal	WF9 × M14 normal	R802 × R75 opaque-2	R802 floury-2	R802 normal	R802 opaque-2	WF9 × M14 normal	R802 × R75 opaque-2	R802 floury-2
Lysine	0.1	0.1	0.1	0.1	0.1	0.1	0.3	0.4	0.4
Histidine	0.7	1.1	0.9	1.0	1.6	1.7	3.2	2.4	1.4
Arginine	1.0	1.1	1.4	1.2	1.6	1.3	2.2	1.9	1.5
Aspartic acid	5.4	4.8	5.0	5.5	3.6	3.6	2.7	3.6	4.0
Threonine	2.9	2.9	2.8	2.9	2.9	3.2	3.4	3.4	3.2
Serine	5.7	6.2	6.0	5.9	5.0	5.1	5.2	5.8	5.5
Glutamic acid	21.0	20.4	20.3	20.6	19.2	18.9	18.5	18.8	18.6
Proline	10.2	11.3	11.6	10.5	12.6	13.6	15.1	14.0	12.3
Glycine	2.1	2.4	2.6	1.9	5.2	5.6	5.9	5.5	4.5
Alanine	14.2	13.2	12.8	13.7	12.1	10.9	9.7	10.6	11.2
Half-cystine	0.1	0.5	0.5	0.3	1.7	1.7	2.9	1.7	2.6
Valine	4.8	3.9	3.5	4.0	3.8	4.1	4.8	4.4	4.1
Methionine	0.3	1.2	0.4	1.0	5.6	5.5	3.9	3.4	5.6
Isoleucine	4.2	3.8	4.1	4.1	2.7	2.8	2.5	2.6	3.0
Leucine	19.5	18.8	19.2	19.0	14.6	14.0	13.4	14.1	15.0
Tyrosine	2.9	3.5	3.4	3.2	4.2	3.4	3.7	3.8	3.6
Phenylalanine	4.8	5.0	5.4	4.9	3.5	4.2	2.7	3.8	3.6

Table VI. Amino Acid Composition of Glutelin and of Residue Proteins from Endosperms of Normal, Opaque-2, and Floury-2 Inbreds and Hybrids

	μmol/100 μmol									
	Glutelin					Residue				
	R802 normal	R802 opaque-2	WF9 × M14 normal	R802 × R75 opaque-2	R802 floury-2	R802 normal	R802 opaque-2	WF9 × M14 normal	R802 × R75 opaque-2	R802 floury-2
Lysine	4.7	4.7	4.5	5.5	5.5	4.5	6.0	3.4	4.7	4.7
Histidine	3.5	3.2	4.1	3.7	3.0	2.7	2.9	3.2	3.3	2.8
Arginine	4.2	4.3	4.0	4.7	4.5	4.2	4.7	4.1	5.4	4.3
Aspartic acid	7.3	7.9	7.1	8.2	8.8	8.1	7.9	6.8	7.3	8.1
Threonine	4.3	4.5	4.5	4.3	4.5	4.4	4.6	4.2	4.3	4.5
Serine	5.2	5.5	5.2	5.1	5.6	5.4	5.7	5.1	5.6	5.6
Glutamic acid	14.1	13.2	14.6	13.0	13.3	12.0	11.0	13.3	13.0	12.5
Proline	9.6	9.5	10.5	8.3	8.1	9.1	7.7	13.0	9.8	7.3
Glycine	8.5	8.8	8.2	8.3	8.0	8.9	10.2	9.0	10.0	8.6
Alanine	8.6	8.6	8.5	8.7	9.2	10.4	9.9	9.4	9.3	10.6
Half-cystine	1.4	0.8	0.6	0.6	0.5					
Valine	7.4	7.7	7.3	7.6	7.2	7.7	8.0	7.5	7.5	7.7
Methionine	1.7	1.8	1.7	1.9	1.4	2.0	2.0	1.5	2.0	2.0
Isoleucine	4.2	4.4	4.2	4.6	4.5	4.3	4.3	3.7	3.8	4.3
Leucine	9.1	9.0	9.3	9.2	9.5	10.4	9.0	10.2	8.9	10.8
Tyrosine	2.8	2.7	2.7	2.6	2.9	2.2	2.1	2.0	1.9	2.2
Phenylalanine	3.3	3.4	3.1	3.7	3.5	3.8	3.8	3.6	3.3	4.0

Table VII. Amino Acid Composition of Osborne Glutelin and Its Alcohol Soluble and Insoluble Components from the Endosperm of the Normal Hybrid WF9 × M14

	μmol/100 μmol		
	Osborne glutelin	60% 2-propanol-insoluble	60% 2-propanol-soluble
Lysine	2.4	3.7	0.5
Histidine	2.4	2.4	1.8
Arginine	3.0	4.3	2.0
Aspartic acid	5.9	8.1	3.7
Threonine	4.0	4.8	3.5
Serine	5.8	6.3	5.6
Glutamic acid	16.6	14.4	19.5
Proline	10.7	7.8	13.1
Glycine	6.7	8.2	5.4
Alanine	9.9	9.0	11.5
Half-cystine	0.9	0.6	0.7
Valine	5.8	7.0	4.5
Methionine	3.3	2.7	3.7
Isoleucine	3.5	4.4	2.8
Leucine	11.8	9.7	14.5
Tyrosine	3.4	2.9	3.8
Phenylalanine	3.9	3.7	3.5
% of total N	100	55	45

60% 2-propanol, pH 6, and a subfraction soluble in 2-propanol. The amino acid compositions are given in Table VII. Clearly, the component insoluble in 2-propanol at pH 6 resembles the glutelins in Table VI, while the protein soluble in 2-propanol resembles zein-2. In this case the disulfide bonds had not been reduced by 2-mercaptoethanol, though possibly they had been broken by NaOH. Methionine is high in the original glutelin and is even higher in in the 2-propanol soluble fraction. Histidine is one amino acid which did not follow the expected pattern.

The residue left after the extraction of the other protein fractions has an amino acid composition quite similar to glutelin (Table VI). The most variable amino acid was lysine (3.4–6.0%). The residue may be unextracted glutelin plus variable amounts on globulins and albumins associated with starch and cell debris.

Polyacrylamide Gel Electrophoresis. The samples assayed for amino acid composition were not available for gel electrophoresis. The samples presented here were isolated in similar fashion, and were selected to provide a very general

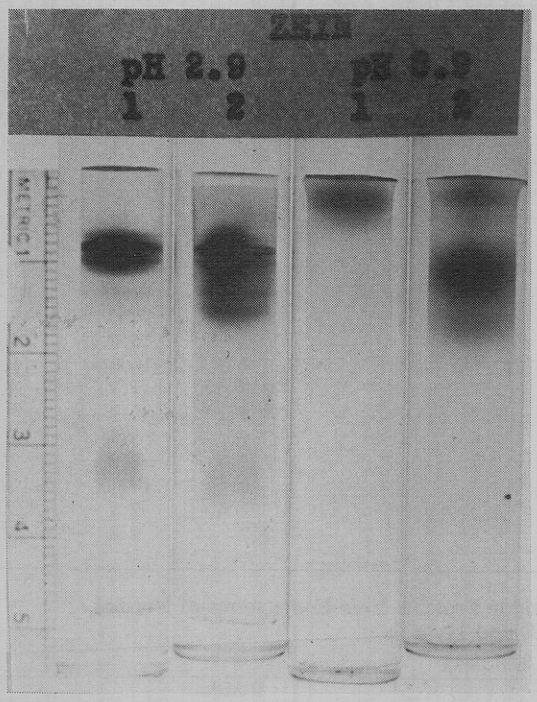

Figure 1. Polyacrylamide gel electrophoresis of zein-1 and zein-2. The zein was extracted from WF9 × M14 endosperm. Electrophoresis was run for 2 hr at pH 2.9 and for 1 hr at pH 8.9. The negative electrode was at the bottom for pH 2.9 and at the top for pH 8.9. The grey bands below 3 cm at pH 2.9 are artifacts, possibly from a reagent impurity

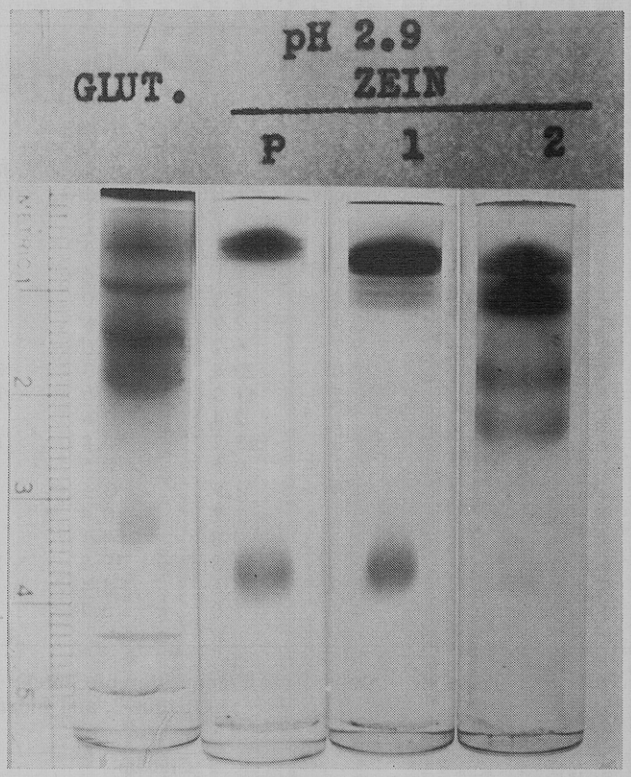

Figure 2. Polyacrylamide gel electrophoresis of glutelin, purified zein, zein-1, and zein-2. The samples were obtained from different endosperms and were run at different times, each for about 70 min. Approximately 75 μg of the purified zein was placed on the gel

picture of the differences between proteins not present in zein-1. Zein-1 separated into two major and two minor bands which migrated between 7 and 11 mm, at pH 2.9. Two additional minor bands were also noted at 13 and 15 mm. The zein-2 preparation in Figure 1 possessed major bands similar to those of zein-l pH 2.9, but the bands at 13 and 15 mm were much more intense than in zein-1. Faint bands could be seen at 26 and 31 mm. At pH 8.9 the zein-1 consisted of two poorly defined, slowly moving bands. These were in the region occupied by bands of purified zein (not shown). The zein-2 extract also contained bands in this region, but in this case the major bands moved more rapidly. Other preparations of zein-2 did not have as much fast-moving protein. The amount of fast-moving protein is over-estimated by amido black staining, for the more highly charged, faster moving proteins will absorb more of the dye (Lawrence *et al.*, 1970).

The samples in Figure 2 were selected to illustrate different protein fractions and are not from the same run. The purified zein (P) separated into three slow-moving bands which corresponded to similar sets of bands in both the zein-1 and zein-2 preparations. The zein-1 contained some minor, slightly faster protein bands which corresponded to a set of major bands in the zein-2 extract. Each of the bands in each set are quite sharp, though this cannot be seen well except when less protein is placed on the gels. The zein-2 contains, in addition, two broad, fast moving bands at 18 and 22 mm. It is possible that these bands are the same as the bands at 14 and 18 mm in the glutelin gel. The glutelin also contains slow moving bands which could be related to zein. However, the large differences in amino acid composition suggest that the correspondence of bands after electrophoresis should not be taken as evidence that the proteins are the same.

DISCUSSION

Advances in our knowledge of storage proteins in plant seeds and in the processes by which they are synthesized depend upon satisfactory methods for separation and identification of the various proteins. The value of using 2-mercaptoethanol is apparent from the similarity in the amino acid compositions of the corresponding protein extracts from different corn genotypes. There are some minor differences which might be related to the different genetic backgrounds, but these differences are small compared to the differences in the sizes of the different protein classes (Table II) or in the differences in the overall amino acid composition of the whole endosperms (Table III). However, the proteins (zein-1, zein-2, and glutelin) are still not homogeneous, as shown by gel electrophoresis (Figures 1 and 2). The proteins which are separated on the gels may have very similar amino acid compositions or they may be closely linked either genetically or during the process of protein synthesis.

Zeins. The fraction which we have called zein-1 is very similar in amino acid composition to the zeins extracted by 70–85% ethanol at room temperature by a number of workers (Boundy *et al.*, 1967; Jimenez, 1968; Landry and Moureaux, 1970; Mosse *et al.*, 1966; Paulis *et al.*, 1969; Waldschmidt-Leitz and Kling, 1967). Thus the preparation of a "pure" zein uncontaminated by other proteins does not appear to be a problem. However, not all of the zein is extracted by this procedure. The fraction which we have called zein-2 contains, we think, a large portion of the previously

unextracted zein, plus variable amounts of other proteins. This "alcohol-soluble glutelin" was designated "G_1" by Moureaux and Landry (1968), who first noted the value of including 2-mercaptoethanol in the solvent medium. They have described the protein as having the characteristics of a prolamine (Landry and Moureaux, 1970). To avoid confusion, we wish to designate this protein by its characteristics, rather than by the protein fraction, glutelin, in which it is often a contaminant. The amino acid composition of the zein-2 reported here is very similar to that of the G_1 protein of Landry and Moureaux (1970).

Zein-2 has not been extracted as a separate fraction elsewhere, but close examination of the amino acid compositions of zeins extracted at 60° C (Mosse *et al.*, 1966) or by alcohol containing 0.5% sodium acetate (Paulis *et al.*, 1969) suggests that these proteins contained some zein-2. In another approach to protein fractionation, Christianson *et al.* (1969) isolated protein bodies from immature endosperms by density-gradient centrifugation. The alcohol-soluble protein from the free protein bodies had an amino acid composition shifted from the classical zein type toward the zein-2 composition which we found.

The high methionine content of zein-2 is of interest from a nutritional point of view. However, we find that high methionine is associated with low lysine. If most of the methionine were to be found in one of the nonzein proteins in the zein-2 fraction, it must make up a large part of this protein, for the low lysine content of zein-2 suggests that there are only small amounts of proteins with high lysine composition. Rather, it may be more reasonable to postulate that most of the methionine is present in different low lysine proteins which make up a large part of the zein-2 fraction. These might be the proteins which are present in very small amounts in zein-1 preparations but in much larger amounts in zein-2 preparations (Figures 1 and 2). Houston and Mohammed (1970) isolated a globulin with a low lysine and a high methionine content from rice endosperm. Methionine is easily oxidized during protein hydrolysis, so that low values for methionine in the literature may not mean that it was not present. Mosse *et al.* (1966) found 2.5% methionine in zein extracted at 60° C in contrast to 1.6% found after 25° C extraction. We found 3.3% methionine in the "Osborne" glutelin, while Jimenez (1968) reported 4.2% (g/100 g) methionine in the glutelin insoluble at pH 6. Paulis *et al.* (1969) also found 3.2% methionine in some of their glutelin fractions.

Glutelins. The amino acid composition of glutelins is quite variable, as reported by different laboratories, presumably as a result of different levels of zein contamination. The lysine content of glutelin will be lowered in proportion to the zein contamination. Thus we note 1.1% (Dalby, 1966), 1.9% (Boundy *et al.*, 1967), 2.3% (Paulis *et al.*, 1969), or 2.4% (Table VI) lysine in glutelin after ordinary alcohol extraction of zein. Hot alcohol extraction left 3.0% lysine (Mosse *et al.*, 1966) in glutelin, while acetate–alcohol and enzyme treatments gave glutelins ranging from 2.7 to 4.3% lysine (Paulis *et al.*, 1969). After zein-2 was removed with mercaptoethanol, we recovered glutelins with 4.5 to 5.5% lysine, depending on the genotype involved (Table VI).

Landry and Moureaux (1970) further subdivided glutelin into fractions G_2 and G_3, extracted without and with detergent after extraction of prolamines with 2-mercaptoethanol in alcohol. G_2 and G_3 had the following characteristic amino acid contents, in mol/100 mol: lysine, 1.1 and 5.1; histidine, 6.2 and 2.5; aspartic acid, 2.1 and 8.0; glutamic acid, 19.8 and 12.2; and proline 19.5 and 6.8. With the exception of histidine, our subfractions of an Osborne glutelin (Table VII) showed similar trends for these amino acids, even though the alcohol-soluble fraction appeared to contain zein-2 as well. Jimenez (1968) divided glutelin on the basis of solubility in aqueous solution at pH 6 after alkaline extraction. The soluble fraction resembled the G_2 amino acid composition and the insoluble fraction the G_3 composition. Christianson *et al.* (1969) obtained two alkali-soluble fractions after density gradient centrifugation. The Zone IV fraction resembled the G_2-glutelin and the Zone II fraction resembled the G_3-glutelin. Thus, four different methods which subdivided the "glutelin" yielded two fractions with characteristically different amino acid compositions. The proportions of these fractions may be affected by the efficiency of the extraction of proteins from the residue, as well as by possible genetic control. Gel electrophoresis of different glutelins (Figure 2; Christianson *et al.*, 1969; Paulis *et al.*, 1969) revealed a number of bands, suggesting that there are additional fractions to be isolated and identified.

Mutant Endosperms. The principal effect of the opaque-2 gene is to depress zein synthesis, while the increased production of other proteins should be considered as a secondary effect. Zein-1 was greatly depressed, while zein-2 was much less affected (Table II). The opaque-2 mutation also has only a small effect on the amount of additional zein extracted by hot alcohol (Mosse, 1966) or by including sodium acetate in the alcohol (Paulis *et al.*, 1969). Electrophoretic studies suggest that a portion of a zein-2 fraction may be zein-1, but the amino acid composition of zein-2 fractions suggest little change in relative proportions of the different proteins in this fraction in the opaque-2 endosperm. However, these conclusions must be tentative until quantitative studies can be made of the proteins separated by electrophoresis and the proteins can be further identified by amino acid analysis and other criteria.

Although the floury-2 sample did have a lysine content higher than the normal endosperm (Table III), the proportions of the different proteins were not greatly changed (Table II). We have no explanation for this, except for possible losses during the successive extraction procedures. This particular sample of floury-2 differs greatly from the one reported by Mosse (1966), which had a high nonprotein nitrogen content. This contrast is more striking when we consider that the opaque-2 version of R802 did have a high nonprotein nitrogen content. Different corn varieties differ considerably in total protein content and in alcohol-soluble protein content (Wolf *et al.*, 1969). It is not unexpected to find that different varieties respond differently to mutations which drastically change the synthesis of a major storage protein.

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