Comparison of Glutelin Proteins in Normal and High-Lysine Corn Endosperms

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Purification, structure, and amino acid composition of the glutelins in normal and high-lysine corn endosperms were investigated to determine whether differences in these proteins occur in the two varieties. After removal of saline-soluble proteins, endosperm meals were exhaustively extracted with solutions of either 70% ethanol or 70% ethanol containing 0.5% sodium acetate. The latter solution removed more zein than 70% ethanol alone. Portions of meal residues were then extracted with 0.2% sodium hydroxide to yield glutelin, which appeared partially degraded. To obtain undegraded glutelin, another residue portion was dispersed in dimethyl sulfoxide solution and its starch digested with α-amylase. After this glutelin had been reduced and alkylated, additional protein was removed by 70% ethanol extraction. Amino acid composition of the native glutelins and starch-gel electrophoretic patterns of the reduced and alkylated proteins were compared. These data indicated that glutelins of the high-lysine and normal hybrids differed only slightly and that major differences noted in some preparations may result from a greater association of normal glutelin with zein or zein-like proteins.

In the mutant corn variety, *opaque-2*, shown to be high in lysine content by Mertz *et al.* (1964), glutelin is the major protein. In contrast, in normal corn endosperm zein is the predominant protein. Glutelin is crosslinked through disulfide bonds, present in cystine residues, which render the protein insoluble in both saline and 70% ethanol solutions (Nielsen *et al.*, 1968). A major question concerning the genetic changes that give rise to the high-lysine property is whether only the amounts of the different protein classes are changed or whether the protein classes have also been altered in composition and structure.

The classical procedure for isolation of corn proteins devised by Osborne and Mendel (1914) consists of successively extracting meal with water, saline, and alcohol solutions to remove albumins, globulins, and zeins and then removing glutelin with an alkaline solution. Lloyd and Mertz (1958) used alkaline-copper sulfite solution to separate most of the corn protein. From this extract they separated glutelin by a modified Osborne-Mendel fractionation. Such procedures may cause glutelin degradation, including cleavage of disulfide bonds. Amino acid analyses of the proteins obtained by these two methods of isolation led to conflicting conclusions about the similarity of glutelins from normal and high-lysine corn endosperms (Jiménez, 1966; Mertz *et al.*, 1967).

To reconcile these differing results, ways to isolate purified native proteins were investigated. The method of Concon (1966) who used α-amylase to remove starch was modified. Considerable attention was directed to the problem of zein contamination and its possible interaction with glutelins to give variable analyses.

EXPERIMENTAL

Treatment of Grain. Grain from a high-lysine hybrid containing *opaque-2* genes, and from the normal counterpart corn (R801 × Oh7N), 1966 harvest, was supplied by D. E. Alexander of the University of Illinois. The kernels were soaked in water for several minutes at room temperature before being manually dehulled and degermed. The air-dried endosperms were ground to pass through a 100-mesh sieve and partially defatted with petroleum ether at 5° C. (Boundy *et al.*, 1967).

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Fractionation of Proteins. Saline-soluble proteins were removed by stirring the defatted meal twice for 1 hour at 5° C., in 0.5*M* NaCl; first a 5 to 1 (v. per w.) solvent to meal ratio was used, followed by a 5 to 2 ratio. Each suspension was centrifuged at 2300 × G at 5° C. for 15 minutes. Proteins were purified by precipitating them from the supernates of both extractions by slowly adding, with stirring, $(NH_4)_2SO_4$ to saturation. The precipitated protein was separated, washed twice with distilled water at 4° C., and redissolved in additional cold 0.5*M* NaCl. Water washings and 0.5*M* NaCl protein solutions were combined and dialyzed at 4° C. The contents of the dialysis bags were centrifuged and the precipitated globulins and the supernates containing the albumins were freeze-dried separately.

Meal residues remaining after the 0.5*M* NaCl extraction were washed free of salt with water. Zein was extracted from the residue by stirring with a 5 to 1 (v. per w.) solvent to meal ratio of either 70% ethanol or 70% ethanol containing 0.5% sodium acetate at room temperature for 3 hours, three times. Earlier, Nagy *et al.* (1941) demonstrated that the addition of 0.5% sodium acetate to an alcohol solution increased extraction of zein from commercial corn meal. After each extraction the suspensions were centrifuged at 2300 × G for 20 minutes. All supernates were combined and dialyzed against water. After dialysis the water-insoluble zein was removed by centrifugation and freeze-dried.

Residual meals were then washed free of solvents with water and also freeze-dried. To obtain the glutelin protein, portions of these residues were either extracted with alkali or treated with α-amylase.

Glutelin was extracted by stirring the dry residues three times with fresh 0.2% NaOH in a 5 to 1 solvent to residue ratio (v. per w.) at 3° C. for 2 hours. After each extraction the protein was precipitated from the separated supernate by adjusting it to pH 5.9 with 10% acetic acid. The precipitated glutelin was washed several times with cold water and then lyophilized.

Improved glutelin isolation involved removal of starch from the meal. Previously extracted dry meal residue, 10 grams, was blended for 30 minutes with 150 ml. 90% dimethyl sulfoxide (DMSO) to disrupt thoroughly starch granules. This suspension was then diluted with 0.04*M* glycerol phosphate buffer pH 6.9 to 50% DMSO (A. C. Beckwith, 1968). About 8 mg. of α-amylase (Mann hog pancreas, twice recrystallized) was added. The mixture was incubated with agitation at 28° C. for 20 hours. Digestion of starch was

Table I. Relative Amounts of Protein Fractions in Endosperm of Normal and High-Lysine Corns

Fraction	Proteins	Total Protein, % Normal	Total Protein, % High Lysine
0.5*M* NaCl	Albumins and globulins	7.8	20.2
Ethanol			
70% EtOH	Zeins	37.6	14.6
70% EtOH–0.5% Na acetate	Zeins	45.8	24.4
0.2% NaOH			
After EtOH	Glutelins	43.6	53.2
After EtOH–0.5% Na acetate	Glutelins	37.5	46.2
Total soluble			
With EtOH	...	89.0	88.0
With EtOH–0.5% Na acetate	...	91.1	90.8

Table II. Comparison of Amino Acids in 70% Ethanol and 70% Ethanol-Acetate Extracted Zeins

Amino Acid	mMoles/16 Grams *N*: Alcohol *N*[a]	Alcohol O_2[b]	Alcohol-Acetate *N*	Alcohol-Acetate O_2
Lysine	0.6	1	1	0.7
Histidine	8	8	28	16
Ammonia	148	183	200	197
Arginine	10	11	12	12
Aspartic acid	41	38	36	36
Threonine	24	24	29	28
Serine	52	52	52	52
Glutamic acid	166	157	180	174
Proline	94	102	70	117
Glycine	17	20	33	32
Alanine	110	108	100	95
Valine	31	25	31	32
Methionine	10	5	10	9
Isoleucine	31	31	30	29
Leucine	151	157	148	149
Tyrosine	31	30	32	31
Phenylalanine	43	44	43	41

[a] *N* = normal corn endosperm.
[b] O_2 = *opaque-2* high-lysine corn endosperm.

monitored by testing 0.1-ml. aliquots at intervals with KI_3 solution (McCready and Hassid, 1943). After digestion was complete, the suspension was diluted 1 to 1 (v. per v.) with distilled water and centrifuged at 2300 × G for 20 minutes. After several washings with water to remove solvent and residual traces of enzyme, the precipitated material was lyophilized.

Reduction and Alkylation. Disulfide groups in portions of the isolated proteins were reduced with a 100-fold excess of mercaptoethanol over protein cystine sulfur in a 0.01*M* phosphate pH 8 buffer containing 8*M* urea for 16 hours at room temperature (Cavins and Friedman, 1968a). For alkylating sulfhydryls a twofold excess of acrylonitrile over total sulfhydryl was added to the solutions and was allowed to react for 1 hour at room temperature. All solutions were then adjusted to pH 3 with acetic acid, dialyzed against water as before, and lyophilized.

Removal of Additional Alcohol Solubles from Glutelin. Extraction of additional alcohol-soluble protein from the α-amylase prepared glutelin was investigated for both native and alkylated-reduced protein. For this purpose each preparation (60 mg.) was stirred with 2 ml. of 70% ethanol at room temperature for 2 hours three times. Each suspension was centrifuged at 2300 × G for 10 minutes. Nitrogen was determined on the supernates, and precipitates were freeze-dried.

Amino Acid Analysis. Defatted endosperm meal samples (100 mg.) were hydrolyzed by refluxing in 200 ml. of constant boiling HCl for 24 hours. For purified proteins, 10-mg. samples were similarly hydrolyzed in 25 ml. of acid solution. Amino acids in the hydrolyzates were quantitatively determined with a Phoenix automatic amino acid analyzer, Model K-8000 by Benson and Patterson's (1965) accelerated procedure. Tryptophan and cystine were not determined. The results were calculated according to a procedure developed by Cavins and Friedman (1968b). All amino acid compositions were corrected to 100% nitrogen recovery.

Starch-Gel Electrophoresis. Electrophoresis was carried out according to a method described by Paulis and Wall (1969) except when zeins were electrophoresed alone. With zeins a pH 3.8 aluminum lactate buffer (0.08*M* aluminum lactate and 0.04*M* lactic acid) containing 8*M* urea was used for preparing the gels and conducting electrophoresis. These gels were stained for 16 hours with a 0.2% light yellow green S.F. dye solution in 7% acetic acid.

RESULTS AND DISCUSSION

Meal Composition. The defatted *opaque-2* and normal endosperm meals contained 1.2 and 1.5% nitrogen, respectively. Based on a conversion factor of 6.25, these meals contained 7.5 and 9.4% protein. The *opaque-2* endosperm meal protein contained 3.6% lysine, whereas that of the normal, only 2%.

Removal of Nonglutelin Proteins. The amount of protein in each fraction extracted from both high-lysine and normal endosperms is shown in Table I. Extraction with 0.5*M* NaCl removed almost three times more protein from high-lysine endosperm (20.2%) than from the normal (7.8%). The 70% ethanol extracted more than twice as much protein from normal (37.6%) as from high lysine (14.6%) endosperm. These yields for saline and alcohol-soluble proteins are consistent with those reported by others (Jiménez, 1966; Mossé *et al.*, 1966). Further extraction with 70% alcohol at room temperature removed no additional protein.

In our extractions the addition of 0.5% sodium acetate to 70% ethanol increased the amount of protein extracted from both normal and high-lysine endosperms by almost 10%. Amino acid composition of the zein extracted with alcohol-acetate differs little from that obtained with 70% alcohol solution, except for histidine, glutamic acid, and glycine (Columns 1 and 2, Table II). The starch-gel electrophoretic patterns of the 70% ethanol and 70% ethanol-acetate were similar except that a fast moving band was more prominent in the alcohol-acetate preparation (Figure 1). Apparently, either due to the slight alkalinity (about pH 7.9) or increased ionic strength, 70% ethanol containing 0.5% sodium acetate is effective in extracting more zein and possibly a small amount of other protein from both high-lysine and normal corn endosperms than 70% ethanol alone.

Comparison of Glutelins. The amount of protein extracted with 0.2% NaOH solution from the residues is given in Table I. The glutelin represents that protein precipitated by neutralization of the 0.2% NaOH extract and accounts for 60% of the total residue nitrogen. Protein present in the supernate was not recovered in this study. With 0.2% NaOH about 7% more protein was separated from the ethanol-extracted residues than from the ethanol-acetate-extracted ones. This additional protein was probably zein, which was not previously extracted by ethanol but which was removed with alcohol-acetate. From both the ethanol- and ethanol-acetate-extracted residues, 0.2% NaOH removed about 9% more protein from high-lysine than normal corn. The per-

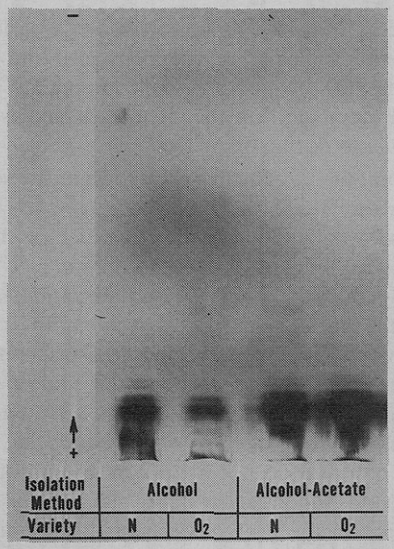


Figure 1. Starch-gel electrophoretic patterns of 70% ethanol and 70% ethanol-0.5% NaOAc extracted zeins from high-lysine (O_2) and normal (N) corn endosperms

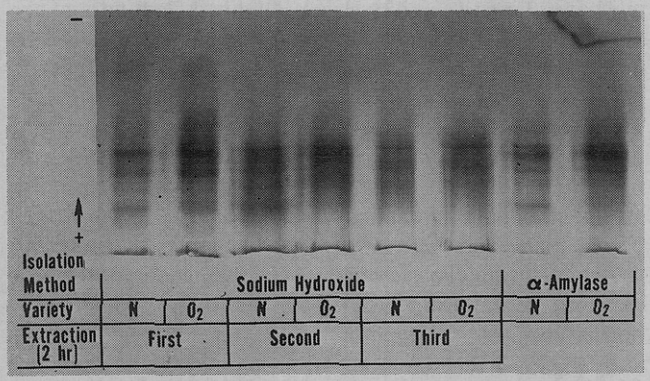


Figure 2. Starch-gel electrophoretic patterns of reduced and alkylated glutelins isolated by 0.2% NaOH extraction and enzyme treatment from high-lysine (O_2) and normal (N) endosperms

Table III. Comparison of Amino Acids in Various Glutelins from Normal (N) and High-Lysine (O_2) Corn

(mMoles/16 Grams N)

	Preparation							
					Enzyme			
	Sodium Hydroxide				Native		Extracted Alk-Red[a]	
Amino Acid	N[b]	O_2[b]	N	O_2	N	O_2	N	O_2
Lysine	18	24	21	35	27	32	29	31
Histidine	18	18	21	26	30	32	27	29
Ammonia	202	214	198	136	127	119	124	127
Arginine	30	27	28	35	31	33	31	33
Aspartic acid	*49*	*53*	*54*	*66*	*48*	*51*	*49*	*49*
Threonine	34	33	35	39	29	40	35	37
Serine	47	44	47	48	46	43	44	45
Glutamic acid	131	113	130	121	128	122	122	118
Proline	73	72	76	72	90	87	96	100
Glycine	62	62	61	70	72	74	67	73
Alanine	79	69	76	74	74	68	70	67
Valine	45	52	44	55	51	54	57	62
Methionine	25	14	25	17	17	19	16	13
Isoleucine	29	27	32	34	31	32	29	30
Leucine	85	71	87	79	82	78	74	74
Tyrosine	31	23	30	25	25	23	21	21
Phenylalanine	27	25	24	26	26	25	23	23

[a] Alk-Red = Reduced and alkylated enzyme-treated glutelin which was extracted with 70% ethanol.
[b] Previous extraction, alcohol; all the rest alcohol-acetate.

centage of total protein extracted by the combined saline, alcohol, and sodium hydroxide solvents was about the same from both high-lysine and normal meals (Table I).

A comparison of the effects of either previous alcohol or alcohol-acetate extraction on the amino acid compositions of glutelins is shown in Table III. Lysine content of *opaque-2* glutelins is greater than that of normal glutelins. Lysine content in glutelins from both varieties is higher in the preparations extracted with alcohol-acetate than in those previously extracted with a 70% alcohol solution. Since the zein content of high-lysine corn is lower than that of normal, less zein may be retained in high-lysine meal. By removing more zein, the alcohol-acetate extraction increases the level of lysine in the glutelins. In general, all the glutelins have amino acid compositions intermediate between those of zeins (Table II) and those reported for corn albumins and globulins (Paulis and Wall, 1969).

Since alkali may degrade glutelin, other methods were sought to separate the protein from starch. Concon (1966) solubilized starch in corn meals after gelatinizing it by heating. By using 90% DMSO as the dispersing agent (A. C. Beckwith, 1968), we gelatinized starch at room temperature thereby reducing the possibility of protein denaturation. Subsequent digestion of the starch with α-amylase in 1 to 1 DMSO to phosphate buffer yielded an insoluble material consisting of 65% protein and representing about 80% of the total nitrogen in the meal residue. Losses of protein due to protease in the enzyme preparation was unlikely since the α-amylase was highly purified (Caldwell *et al.*, 1952). Protein losses from the insoluble residues following amylolysis, may be due to release of soluble protein entrapped in the starch granule.

Table III compares the amino acid composition of the glutelins from normal and high-lysine corns prepared by both 0.2% NaOH and α-amylase. Both high-lysine glutelins contain more lysine than the normal corn glutelins. In contrast the normal glutelin is higher in proline and leucine than the glutelin prepared by the same method from high-lysine corn. Since these nonpolar amino acids are prominent in zein, their relative amounts suggest a greater association of zein-like protein with normal glutelin.

Significant variations were noted between glutelins prepared by the two methods. The difference between lysine contents of glutelins from normal and high-lysine endosperms prepared by the sodium hydroxide extraction is greater than that between glutelins from the two genotypes prepared by the enzyme technique (Table III). This variation suggests that protein deficient in lysine is preferentially extracted by sodium hydroxide from the meal. More of this protein is extracted from normal than high-lysine corn.

After stirring 1% suspensions of enzyme-prepared glutelins in 8M urea pH 8 phosphate buffer for 16 hours, 89 to 92% of the proteins remained insoluble in the solvent. When these glutelins were reduced and alkylated in 8M urea-phosphate pH 8 buffer, about 60 to 70% of the material (accounting for 85 to 90% of the total nitrogen of the preparation) was soluble in this buffer or in the pH 3.1 aluminum lactate–8M urea electrophoresis buffer. The fraction soluble in 8M urea phosphate pH 8, when dialyzed against water to remove urea and salt, yielded material after lyophilization consisting of 90% protein (lyophilized weight). Probably the small amount of protein not soluble in urea after reduction and alkylation is not glutelin. Its composition and properties are being investigated.

In Figure 2 are shown starch-gel electrophoretic patterns

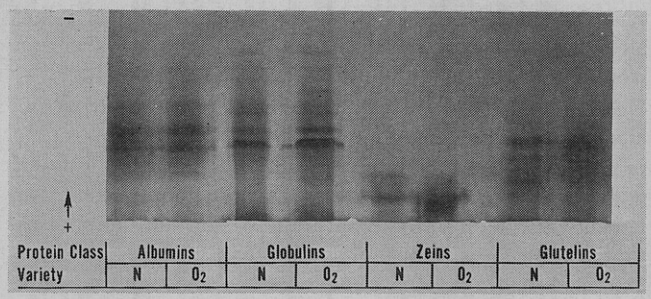
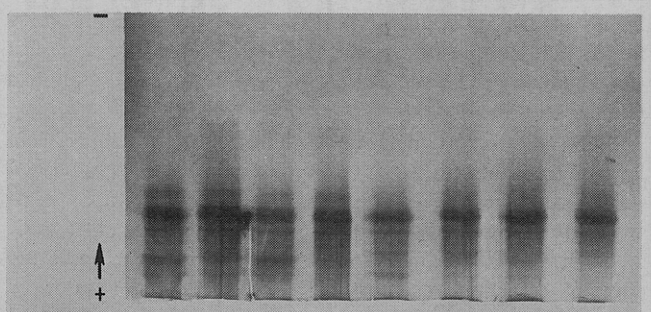


Figure 3. Starch-gel electrophoretic patterns of reduced and alkylated proteins from high-lysine (O_2) and normal (N) endosperms



Variety	Normal				High Lysine			
Prior Extraction	Alcohol		Alcohol-Acetate		Alcohol		Alcohol-Acetate	
Original or Extracted*	Orig.	Ex.	Orig.	Ex.	Orig.	Ex.	Orig.	Ex.

* Orig. are reduced and alkylated enzyme-prepared glutelins. Ex. are reduced and alkylated enzyme-prepared glutelins which were extracted with 70% ethanol.

Figure 4. Comparison of starch-gel electrophoretic patterns of reduced and alkylated glutelins from high-lysine (O_2) and normal (N) corns before and after the glutelins were extracted with 70% ethanol

Table IV. Amounts of Protein Extracted with 70% Ethanol from Reduced and Alkylated Glutelins

Previous Extraction	Glutelin in, %: O_2[b]	Glutelin in, %: N[c]	Total Protein, %: O_2	Total Protein, %: N	Total Extractable Zein[a]: O_2	Total Extractable Zein[a]: N
Native						
Alcohol	2.1	2.6	1.4	1.4	16.0	39.0
Alcohol-acetate	1.6	1.3	0.9	0.6	25.0	46.4
Reduced and alkylated						
Alcohol	12.6	16.4	8.2	8.9	22.8	46.4
Alcohol-acetate	7.8	14.4	4.3	6.7	28.7	52.5

[a] Values total zein from Table I plus this additional protein. [b] O_2 = *opaque-2* high-lysine corn endosperm. [c] N = normal corn endosperm.

of reduced and alkylated glutelins. The native proteins were prepared by extraction for succesive 2-hour intervals with 0.2% NaOH or by enzyme treatment of both high-lysine and normal endosperms. All these native glutelins were almost completely insoluble in the pH 3.1 aluminum lactate–8M urea buffer used for electrophoresis and therefore did not migrate into the gel when subjected to an electrical potential. When the disulfides of the glutelins were reduced and the resulting sulfhydryls alkylated, the protein soluble in 8M urea migrated upon electrophoresis to give distinct stainable bands. These reduced and alkylated proteins were used in all gel electrophoretic studies since they provided information on the properties of the polypeptide components of glutelins and other proteins and on the chemical changes these polypeptides undergo during extraction.

Proteins obtained by further extraction of the residues with fresh volumes of 0.2% NaOH were progressively more degraded as shown by the greater streaking of their electrophoretic patterns (Figure 2). The first 2-hour NaOH glutelin patterns have the least streaking and are quite similar to the patterns of the enzyme-prepared glutelins of the same variety. The greatest difference between patterns of similarly treated glutelins from high-lysine and normal endosperms is the slightly greater concentration of two slower migrating bands in the normal corn glutelin. These bands have the same mobility as zein and do not appear in native protein (which shows no bands upon electrophoresis). Their appearance after reduction of the protein would indicate that some zein or zein-like components are disulfide crosslinked to themselves or to normal glutelin. The occurrence of zein-like bands in reduced glutelin was reported earlier (Boundy *et al.*, 1967; Dimler, 1966).

Starch-gel patterns of all the reduced and alkylated proteins of different solubility classes obtained from normal and high-lysine endosperms are compared in Figure 3. The reduced and alkylated glutelins primarily migrate with electrophoretic mobilities intermediate between those of similarly modified salt-soluble proteins and zeins. It is noteworthy that the fastest alkylated-reduced glutelin bands have mobilities in this solvent similar to the slowest components of the alkylated-reduced albumins and globulins. Again, faint bands, that exhibit the same mobilities as do zeins, are detected in the glutelin patterns. These bands are most prominent in the pattern of the alkylated-reduced glutelin from normal corn.

In Figure 4 the starch-gel electrophoretic patterns of the alkylated-reduced glutelins prepared by amylase treatment from normal corn exhibit a band of high mobility that is absent or in much lower concentration in the patterns of similarly prepared and modified glutelins from the *opaque-2* corn.

When the amylase-prepared native glutelins were extracted with 70% ethanol, little protein was removed (Table IV). In contrast, extraction of the same material after reduction and alkylation yielded considerable protein soluble in 70% ethanol. More protein was eliminated from glutelin prepared from meals previously subjected to only 70% ethanol extraction than from meals that underwent alcohol-acetate extraction. Less protein was extracted from high-lysine than normal glutelins. Since this protein was not extracted from the native glutelin by the 8M urea pH 3.1 electrophoresis buffer and 70% ethanol, but was soluble in both solvents after reduction and alkylation of glutelin, it can be assumed to be disulfide crosslinked to glutelin or to itself. Because of its solubility in 70% ethanol and electrophoretic mobility, this alkylated-reduced component resembles zein.

The last two columns of Table III also indicate that the protein removed by extraction of alkylated-reduced glutelin is probably related to zein as evidenced by the lower content of leucine compared to the other glutelin preparations. This removal of zein-like material further minimizes differences in the amino acid composition between high-lysine and normal glutelins. As shown in Table III, only minor differences in amino acid composition exist between the high-lysine and normal alkylated-reduced glutelins after alcohol extraction.

The starch-gel electrophoretic patterns of these 70%

ethanol-extracted reduced and alkylated glutelins (Figure 4) show a reduction in two slower migrating bands that correspond to zein in mobility.

These results indicate greater similarity of *opaque-2* and normal glutelins, if free zein and disulfide-crosslinked zein-like protein have been extensively removed from the preparations. Mertz *et al.* (1967) found a similar amino acid composition in glutelin fractions of alkaline-copper sulfite extracts of normal and *opaque-2* endosperms. Their findings also indicate that the disulfide crosslinkages of isolated glutelin may have to be broken completely to remove all the zein from glutelin. This crosslinking may have occurred during the earlier saline and 70% ethanol extraction as a result of disulfide interchange. Previous indications of major variations between glutelins of normal and high-lysine corns may have resulted from inadequate prior extraction of zein and may be partly due to the disulfide bonding of some zein-like protein to glutelin. Further fractionation and characterization of the reduced glutelin proteins is in progress. These experiments will investigate possible relationships of the glutelin proteins to globulins, and albumins, and will seek to ascertain further if differences among the protein groups of normal and high-lysine corns are significant.

ACKNOWLEDGMENT

The authors wish to express their appreciation to J. E. Cavins for amino acid analyses and to A. C. Beckwith for information on procedures for the dispersion and hydrolysis of starch in DMSO solution.

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Received for review April 18, 1969. Accepted August 8, 1969. Presented before the Division of Biological Chemistry, 156th Meeting ACS, Atlantic City, N. J., September 1968. Work done at a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture. Reference to a commercial product is not intended to be a recommendation by the Department of Agriculture over similar ones not mentioned.