

Maize Endosperm Proteins That Contribute to Endosperm Lysine Content

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ABSTRACT

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Nonzein proteins were analyzed using SDS-PAGE to study their relationship to lysine level in mature endosperm of three isogenic lines and 29 inbred lines. The majority of nonzein proteins are glutelins that contain a relatively high lysine content. Trinitrobenzene-sulfonic acid was used to visualize high-lysine proteins on nitrocellulose membrane. Seven intense yellow bands (high-lysine proteins) were found in the glutelin fraction: 35, 43, 48, 52, 84, 92, and 100 kDa. These seven glutelin proteins were also

significantly correlated to each other. Lysine content of the triads correlated with three glutelin proteins (35, 43, and 52 kDa), while lysine content of 29 inbred lines correlated with six glutelin proteins (all except 52-kDa protein). Immunoassays based on these higher lysine glutelin proteins provide an effective screening method in the presence of variable zein levels. Retention or improvement of lysine content is possible using this information in a maize improvement program.

Maize is the basic staple cereal grain for large groups of people in Latin America, Africa, and Asia. Corn provides significant amounts of calories and protein for these people (Mertz 1992). However, maize is relatively low in protein content and has limiting amounts of two essential amino acids, lysine and tryptophan (Bressani 1991). Increased lysine and tryptophan content was observed in the protein composition of the endosperm of the *opaque-2* (*o2*) mutant of maize. The homozygous recessive *o2* gene restricts synthesis of zein that contains little lysine and tryptophan and a pleiotropic increase of the nonzein proteins that contain high lysine and tryptophan levels in endosperm (Habben et al 1993). Agricultural utilization of the *o2* maize has been limited due to reduced yield and undesirable seed characteristics such as soft, chalky kernels that retain moisture and are slow drying at harvest (Lambert et al 1969, Loesch et al 1976). This contributes to greater insect, disease, and aflatoxin susceptibility and mechanical damage during grain harvesting and handling. In the past few decades, some countries that had begun to utilize the *o2* corn have scaled-down the research and production of this type of corn. Farmers in the United States produce about one million tons of Corn's Hi-Lysine hybrid annually out of about 200 million tons of corn produced in United States. Due to poor storage and other processing problems, high-lysine corn stays on the farm to be fed to livestock, mainly growing and finishing pigs.

Through backcrossing and recurrent selection, breeders were able to combine a fairly complex system of genetic modifiers with the *o2* gene to convert normal maize populations into Quality Protein Maize (QPM). The QPM contains modified opaque genes and produces kernels with hard, corneous endosperm (Glover 1992, Mertz 1992). The most difficult problem facing corn protein analysts is providing the breeders with satisfactory procedures for determining the protein nutritional value of corn lines. Breeders must search simultaneously for improved agronomic characters, higher yields, and improved protein nutritional value. Studies using enzyme-linked immunosorbent assay (ELISA) indicated that the 54-kDa protein elongation factor EF1- α significantly correlated to the endosperm lysine content in many normal and *o2* lines (Habben et al 1995, Moro et al 1996, Sun et al 1997). Habben et al (1995), however, showed a negative relationship between EF1- α and lysine content when comparing Tuxpeno QPM (modified Tuxpeno *o2*) and its *o2* counterparts. Sun et al (1997) indicated that the EF1- α level decreased when endosperm matured in both the wild type and the *o2* mutant. Little information is currently available about

the content of individual proteins in mature kernels of QPM that were selected based on lysine content, agronomic traits, yield, and kernel hardness. Characterization of nonzein proteins in flour prepared by drilling a small hole in the endosperm (and then sealed with wax for planting) would be feasible in a QPM breeding program to confirm or predict lysine content. Thus, the objective of this study was to identify specific lysine-enriched nonzein proteins, in addition to EF1- α , in the mature maize endosperm.

MATERIALS AND METHODS

Maize Samples

Mature endosperm tissue of maize cultivars grown at College Station, TX, were analyzed for endosperm protein characteristics: three triads (normal, *o2*, and QPM) of T224, MO17, B73, and 29 QPM inbred lines from the United States, Mexico, and South Africa.

Endosperm Flour Preparation

Dry seeds were steeped in distilled water at 4°C overnight. The germ and pericarp were removed by hand, and endosperm was lyophilized for 48 hr at 70°C and 8 μ m of Hg. Dried endosperm was ground by hand using a pestle and mortar. Lipids were extracted twice in a cold hexane solution for 1 hr each time. Defatted endosperm flour was stored at 20°C. Moisture content of dry endosperm flour was determined by the oven-drying method (Approved Method 44-19, AACC 1995). Crude protein content ($N \times 6.25$) of endosperm flour was determined by the microKjeldahl method with an autoanalyzer system (Method 114/741A, Technicon, Tarrytown, NY).

Protein Extraction

Mature endosperm proteins were extracted and fractionated into three fractions (Landry and Moureaux 1970) with some modification. Proteins were sequentially extracted from endosperm flour (1:6, flour-to-extraction buffer, w/v) as: 1) albumin and globulin, 2) zein, and 3) glutelin. Extraction buffers for the three fractions were: 1) 0.5N NaCl solution for albumin and globulin; 2) 70% ethanol and 2% β -mercaptoethanol (β -ME) for zein; and 3) 1% SDS and 2% β -ME for glutelin. Proteins were extracted twice by shaking the samples and extraction buffers for 1 hr. Albumins and globulins were extracted at 4°C. Zein and glutelin were extracted at 22°C. Mixtures of sample and extraction buffer were centrifuged at 10,000 $\times g$ for 10 min. Supernatants (extracts) from the same fraction were pooled and measured for concentrations by dye-binding method: 1) zeins and glutelins were measured by modified biuret assay using BCA reagent (Pierce, Rockford, IL) using zein as a reference; 2) albumins and globulins were measured by Bradford protein assay (Bradford 1976) using bovine serum albumin as a reference.

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Protein Separation

Each endosperm protein fraction was separated using a modified SDS-PAGE procedure (Hames and Rickwood 1981). Zein extracts were separated using a 12% (w/v) polyacrylamide gel, pH 8.8. The separating gel for albumins, globulins, and glutelins was linear 10–16% (w/v) polyacrylamide gradient gel, pH 8.8. Stacking gel was 4% polyacrylamide gel, pH 6.8. Sample extracts were mixed with the same volume of SDS-PAGE sample buffer (0.125M Tris at pH 6.8, 2% SDS, 0.05% β -ME, 10% glycerol, and bromophenol blue), heated in a boiling water bath for 5 min, and loaded in the sample well. Electrophoresis was conducted at 200V for 45 min. Proteins were stained with 0.1% PhastGel Blue R (Pharmacia, Piscataway, NJ) in 30% methanol and 10% acetic acid, and destained in 30% methanol and 10% acetic acid.

After destaining, images of SDS-PAGE gel were captured using a video camera (Panasonic, OmniMovie HQ, S-VHS). Images of the SDS-PAGE were then analyzed for the protein intensities using an image analysis program (SigmaScan, Jandel, San Rafael, CA).

Direct Staining of Lysine Residues in Proteins

A lysine-staining procedure (Tsai et al 1972) was modified to identify lysine-rich proteins on western blot membrane. Proteins on SDS-PAGE gels were transferred to nitrocellulose membrane (Pierce, Rockford, IL) with Tris-methanol buffer in a semidry electroblotter (Owl Scientific, Woburn, MA). Electroblotting was performed at 400 mA/48 cm² gel for 45 min at 22°C (Gershoni and Palade 1983).

The nitrocellulose membrane was soaked in 20 mL of 0.05M sodium borate in water solution, pH 11. Trinitrobenzene-sulfonic acid (TNBS) (2.0 mL) was then added to the buffer to stain lysine residues of protein. Staining was performed at 40°C for 2 hr. The nitrocellulose membrane was washed with deionized water to remove excessive TNBS. Proteins with higher lysine content were visualized as intense yellow bands on the stained nitrocellulose membrane with the same positions as on SDS-PAGE gel.

Protein Recovery and Analysis of Amino Acid Composition

Proteins suspected to contain higher lysine content were located on the SDS-PAGE gel and cut from the other regions. Proteins were recovered from the gel pieces by electroelutor (model 422, BioRad, Hercules, CA) in a glycine SDS elution buffer (Smith 1984). Amino acid composition analysis of 48-kDa glutelin (one of the proteins suspected to correlate with endosperm lysine content) was conducted by the biotechnology support laboratory, Department of Entomology, Texas A&M University. Proteins were hydrolyzed in hydrochloric acid, and amino acids were analyzed using an HPLC autoanalyzer (Waters PICO TAG amino acids autoanalyzer, Millipore, MA).

TABLE I
Endosperm Protein, Lysine, Albumin + Globulin, Zein, and Glutelin Contents (mg/g of flour) of Triads of T224, B73, and MO17^a

Cultivar	Protein ^b	Lysine	Albumin +		
			Globulin ^c	Zein ^d	Glutelin ^d
T224 N	114.9	1.95	3.8	56.2	28.2
T224 <i>o2</i>	105.1	3.68	6.3	30.6	37.7
T224 QPM	128.1	2.78	2.9	55.4	42.6
B73 N	93.8	1.39	2.2	51.6	30.6
B73 <i>o2</i>	73.1	2.45	4.4	23.8	31.2
B73 QPM	98.8	2.68	4.0	35.6	34.7
MO17 N	91.1	1.27	2.5	61.0	31.6
MO17 <i>o2</i>	89.2	2.15	3.1	49.5	40.9
MO17 QPM	89	2.66	3.5	48.9	44.8
LSD ^e	2.8	0.18	0.5	3.1	3.0

^a Oven dwb. N: normal line; *o2*: opaque 2; QPM: quality protein maize.

^b Measured by micro-Kjeldahl method. Protein calculated as N \times 6.25.

^c Measured by Bradford protein assay (Bradford 1976).

^d Measured by biuret assay (Reinhold 1953).

^e Least significant difference between means.

Statistical Analysis

Analysis of variance was conducted using SAS ANOVA procedure (SAS Institute, Cary, NC). Correlation analysis for data between protein content and endosperm lysine levels was conducted using SAS correlation procedures.

RESULTS AND DISCUSSION

Endosperm Protein Patterns in Normal, *o2*, and QPM Triads

When compared with normal lines within triads of T224 and B73, *o2* mutants had lower endosperm protein content (mg/g of flour), whereas, QPM lines had higher endosperm protein content (Table I). On the other hand, endosperm protein content of MO17 normal, *o2*, and QPM lines were not significantly different. Lower endosperm protein content of *o2* mutant of W22 was also observed by Tsai (1979). The pattern of endosperm protein content of the triads was affected by the distribution of the protein fractions (albumin, globulin, zein, and glutelin) due to the breeding selection.

Both *o2* and QPM lines are mutants that suppress zein synthesis in the endosperm, so zein content of *o2* mutants and QPM of three triads were lower than their normal counterparts, except for zein content of T224 QPM where the reduction of zein was not significant (Table I). However, albumin, globulin, and glutelin content of *o2* mutants and QPM of the triads were higher than that of their normal counterparts, with no significant changes of albumin and globulin content of QPM of T224 and glutelin content of *o2* mutant of B73.

When comparing the fractionated protein content between *o2* mutants and QPM lines of the three triads, a consistent pattern of change was observed only for the glutelin fraction. Glutelin content of QPM lines of three triads were higher than their *o2* counterparts (Table I). Albumin and globulin content of QPM lines, however, were lower or unchanged; whereas, zein content of QPM lines were either higher or unchanged when compared with their *o2* counterparts.

Relationship of Endosperm Protein Patterns and Lysine Content

Because zein has little or no lysine content, decrease of zein content and increase of nonzein content (albumin, globulin, and glutelin) caused higher lysine content in *o2* and QPM endosperm of three triads than in their normal counterparts (Table I). A similar mechanism of higher lysine content in *o2* mutant versus normal W64A was reported by Lee et al (1976).

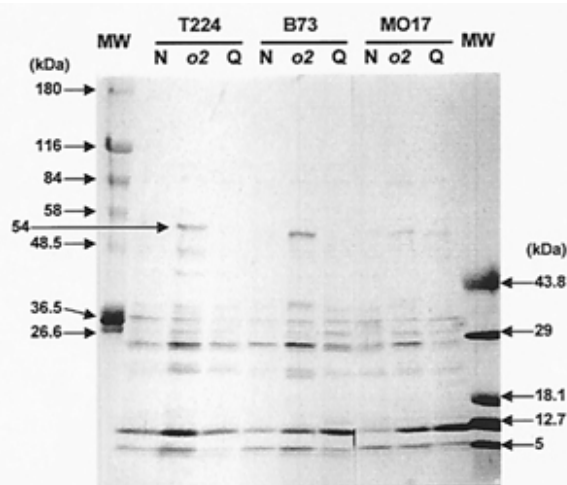


Fig. 1. SDS-PAGE separation of maize albumin and globulin endosperm proteins of T224, B73, and MO17 triads. Each lane contains 12.5 μ L of albumin and globulin extracts (equivalent to albumin and globulin in 2.1 μ g of endosperm flour). Some proteins enhanced in *o2* lines were reduced in Quality Protein Maize (QPM) (arrow). MW: molecular weight marker; N: normal line; *o2*: opaque 2; Q: QPM.

For *o2* mutants and QPM of B73 and MO17, the albumin and globulin content were not significantly different. Higher amounts of lysine in QPM of B73 and MO17 were caused by higher glutelin content (Table I). For T224, higher glutelin content in QPM did not concur with higher lysine content when compared with its *o2* counterpart. Lysine content of QPM of T224 was lower than its *o2* counterpart, as expected from the decreased albumin and globulin content.

Albumins and globulins composed only small amounts of the endosperm protein for all three triads (average of 3.6% of endosperm protein). Some water-soluble (i.e., albumin and globulin) maize endosperm proteins contain significant amounts of lysine such as the 54-kDa EF1- α (11% lysine), trypsin inhibitor (1.5% lysine), and catalase (7% lysine) (Habben et al 1993). However, most of the (lysine-enriched) albumins and globulins (including EF1- α) in *o2* mutant decreased in mature QPM endosperm in our study, except for a 12.7-kDa protein (Fig. 1). Apparently, selection of QPM by conventional breeding program based on endosperm lysine level and agronomic traits did not preserve some specific albumins and globulins originally present in *o2* mutant.

Glutelins, on the other hand, contain an average of 5.4% lysine (Sodek and Wilson 1971) and account for 30–50% of endosperm protein. Some glutelins may also be used as a good indicator of maize endosperm lysine content. Thus, a more intensive search was conducted to identify glutelins that could correlate with endosperm lysine content.

TABLE II
Correlation Between Endosperm Glutelin and Lysine Contents of Three Triads (T224, B73, and MO17) and 29 Inbred Lines

	Glutelin and Lysine Contents (kDa)						
	100	92	84	52	48	43	35
Triads							
<i>r</i>	0.24	0.46	0.58	0.79	0.48	0.77	0.69
<i>P</i> ≤ 0.05	0.54	0.21	0.10	0.01	0.19	0.02	0.04
Inbreds							
<i>r</i>	0.54	0.50	0.70	...	0.38	0.64	0.63
<i>P</i> ≤ 0.05	0.01	0.01	0.01	...	0.04	0.01	0.01

TABLE III
Correlation^a Between Endosperm Proteins of 29 Quality Protein Maize (QPM) Inbreds and Lysine Content

	Glutelin (kDa)					Zein (kDa)				
	92	83	48	43	35	27 ^b	22 ^c	19 ^c	16 ^b	14 ^d
Glutelin (kDa)										
100	0.71 (0.01)	0.84 (0.01)	0.72 (0.01)	0.82 (0.01)	0.72 (0.01)	0.47 (0.01)	-0.23 (0.23)	-0.02 (0.93)	-0.05 (0.79)	-0.25 (0.19)
92		0.75 (0.01)	0.62 (0.01)	0.80 (0.01)	0.84 (0.01)	0.58 (0.01)	-0.47 (0.01)	-0.29 (0.13)	0.27 (0.15)	-0.29 (0.13)
84			0.62 (0.01)	0.84 (0.01)	0.82 (0.01)	0.42 (0.02)	-0.47 (0.01)	-0.21 (0.27)	0.13 (0.52)	-0.33 (0.08)
48				0.59 (0.01)	0.59 (0.01)	0.18 (0.35)	-0.15 (0.45)	0.02 (0.93)	-0.02 (0.91)	-0.14 (0.48)
43					0.88 (0.01)	0.57 (0.01)	-0.49 (0.01)	-0.32 (0.09)	0.15 (0.44)	-0.52 (0.00)
35						0.51 (0.01)	-0.51 (0.00)	-0.41 (0.03)	0.23 (0.22)	-0.43 (0.02)
Zein (kDa)										
27 ^b							-0.47 (0.01)	-0.19 (0.32)	0.57 (0.01)	-0.42 (0.02)
22 ^c								0.81 (0.01)	-0.40 (0.03)	0.71 (0.01)
19 ^c									-0.32 (0.09)	0.77 (0.01)
16 ^b										-0.33 (0.08)

^a Correlation coefficient with *P* value in parentheses (correlation significant at *P* ≤ 0.05).

^b γ -Zein.

^c α -Zein.

^d β -Zein.

Specific High-Lysine Proteins of Maize Triads

Proteins in the glutelin fraction showed seven intense yellow bands on the membrane using the TNBS staining procedure. The intensity of TNBS stained bands on the nitrocellulose membrane, we presume, corresponds to the quantity of these specific proteins and their lysine content. The most abundant stained glutelin (48-kDa) was eluted from SDS-PAGE gel and analyzed for amino acid content. Lysine content of the 48-kDa protein was 5.6 mol%, which is about the same as the average lysine content of glutelin (5.4%) (Sodek and Wilson 1971). Since the yellow color of stained protein bands could not be captured on a photograph, the images of stained proteins are not shown. The molecular weights of seven glutelins, when compared with Coomassie blue stained SDS-PAGE gels, were

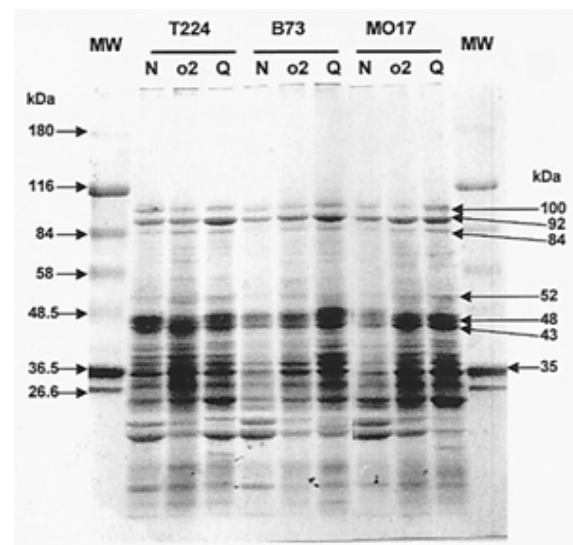


Fig. 2. SDS-PAGE separation of maize glutelin endosperm proteins of T224, B73, and MO17 triads. Each lane contains 12.5 μ L of glutelin extracts (equivalent to glutelin in 2.1 μ g of endosperm flour). Proteins that stained intense yellow with the lysine staining procedure are labeled with arrows. MW: molecular marker, N: normal, *o2*: opaque 2, Q: QPM.

identified as 35, 43, 48, 52, 84, 92, and 100 kDa (Fig. 2). Some proteins of low molecular weight range (5–26 kDa) of the albumins and globulins were lightly stained in a smeared pattern. No yellow-stained proteins were found among the zein proteins.

Effects of Selected Glutelins on Endosperm Lysine Content

Intensity of each protein band from SDS-PAGE was used to estimate protein content. Amounts of seven TNBS-stained glutelins were correlated with the endosperm lysine content of three triads and 29 inbreds. Amounts of 35-, 43-, and 52-kDa glutelins were positively correlated with endosperm lysine content of three triads (Table II). In addition, all glutelins, except 52-kDa glutelin, correlated positively with lysine content for 29 inbred lines ($P < 0.05$).

Sun et al (1997) extracted EF1- α using buffer containing reducing agent β -ME. We suspect that the 52-kDa glutelin in our study was EF1- α . The correlation coefficient of the 52-kDa glutelin to lysine content was the highest among seven glutelins in the three triads. Unfortunately, the 52-kDa glutelin was not detectable in all the 29 QPM inbred endosperm samples using SDS-PAGE with Coomassie blue staining. This caused a lower correlation coefficient with lysine content.

Relationships of individual glutelin and zein intensities of the 29 inbred lines were determined (Table III). The seven glutelins were either negatively ($P < 0.05$) or not correlated with 22- and 19-kDa α -zein, 16-kDa γ -zein, and 14-kDa β -zein. In other words, increases in glutelin content corresponded to decreases or no change in most zein contents. The 27-kDa γ -zein of the 29 inbreds was significantly correlated ($r = 0.64$, $P < 0.05$) with endosperm lysine content and with five of six glutelins measured ($r > 0.42$, $P \leq 0.02$). Larkins and Lopes (1992) and Moro et al (1995) also observed similar results and hypothesized that since γ -zein contains more cystine ($\approx 7\%$) and is located in the periphery of protein bodies, γ -zein may contribute to disulfide bonding with matrix proteins (glutelins). Such association could affect the quantitative relation between 27-kDa γ -zein and glutelins.

Glutelins were positively correlated ($P < 0.05$) to each other for the 29 inbred lines (Table III). In other words, an increased quantity of one glutelin in maize endosperm corresponds to increases in other six high-lysine glutelins. This suggested that these glutelins could be used to select for lysine content in maize.

Potential problems using glutelins to predict lysine content include measurement of free lysine and proportion of zein to glutelin. Because some lysine exists in free amino acids as well as in nonzein proteins, the correlation of lysine with glutelins does not account for the free lysine that was extracted with the albumins and globulins. Zein content (especially α -zeins) correlates negatively with lysine and glutelin content. When cultivars have large variance in zein content, it is not possible to differentiate between increased amounts of glutelin or normal levels of glutelin with higher lysine content in the endosperm. If interference factors are excluded, a higher correlation between high-lysine proteins and endosperm lysine content is expected. The amount of EF1- α explained $>90\%$ of the variability in lysine content (Habben et al 1995). This may have been due to the systematic reduction of zein in $\alpha 2$ lines from their normal counterparts. The EF1- α and lysine content was negatively related for the only QPM and its $\alpha 2$ counterpart (Tuxpeno) (Habben et al 1995). In this study, a significant correlation between lysine-enriched glutelin and endosperm lysine content was observed, even though amounts and patterns of zeins varied in triads and inbred QPM lines (data not shown).

CONCLUSIONS

The main source of lysine in the endosperm is nonzein proteins. The higher lysine content of $\alpha 2$ and QPM mutants is the result of two mechanisms: decreases of one or more zeins and increases of several nonzein proteins. The quantity of 52-kDa glutelin (suspected EF1- α protein) in QPM lines did not correlate to endosperm

lysine content of inbred QPM lines. Six glutelins contained more lysine (reacted with TNBS) and correlated with higher endosperm lysine content. Each high-lysine glutelin was positively correlated to the other high-lysine glutelins. Because these proteins are present in measured QPM lines, an increase of their level is not likely to cause adverse effects on kernel or agronomic traits. Thus, immunoassays specific for one or more of high-lysine glutelins could be utilized to screen endosperm samples to monitor lysine content of mature kernels in a maize breeding program.

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