Zein Composition in Hard and Soft Endosperm of Maize[1]

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ABSTRACT

Maize protein composition and distribution may directly influence endosperm texture and physical properties. To test this hypothesis, we compared compositions of alcohol-soluble proteins in maize endosperm from the hard and soft fractions of eight normal genotypes. Kernels were hand-dissected to obtain fractions differing in texture. Endosperm fractions were extracted with a solution containing alcohol, reducing agent, and sodium acetate and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by reversed-phase high-performance liquid chromatography. We found more (an average of 3.3 times more) α-zeins (19 and 22 kDa) in hard endosperm fractions than in soft endosperm fractions. In contrast, soft endosperm fractions contained nearly twice as much 27-kDa γ-zein (based on percent) than did hard endosperm fractions. Thus, distribution of the various types of zeins was not uniform throughout the maize endosperm. Our results suggest that the zein composition of protein bodies in normal maize kernels may be correlated with texture of the endosperm from which the sample was obtained.

Zeins, the alcohol-soluble maize endosperm storage proteins, are located within protein bodies and are products of multigene families (Lending and Larkins 1989). Different nomenclatures have been proposed to distinguish the various types of zeins (Landry and Moureaux 1970, Paulis and Wall 1977, Wilson 1991). Zein classes α, β, and γ are based on genetic classes determined by sequence homology (Geraghty et al 1982, Pedersen et al 1986, Prat et al 1987) or differential solubility in aqueous alcohol solutions (Esen 1987).

Lending and Larkins (1989) proposed a model of protein body development based on immunolocalization techniques using light and electron microscopy. In the initial stages of kernel development, β- and γ-zeins are distributed throughout small protein bodies, with little or no α-zeins present. In the final stages of protein body maturation, α-zeins fill most of the core of the protein body, which is surrounded by a thin layer of β- and γ-zeins.

Maize kernels frequently contain both horny and floury endosperm, with the periphery of the kernel containing the horny endosperm. Various terms are often used interchangeably in describing kernel properties: *hard, vitreous, translucent*, and *horny* are used as synonyms as are *soft, floury*, and *opaque*. To be accurate, words describing texture should be used only if a hardness measurement has been made (Hoseney 1986). In this study, resistance to hand drilling was the criterion used.

Various studies have described differences between these two types of endosperm. Cells in the floury portion are typically larger and have thicker cell walls than those in the horny portion (Wolf et al 1952). Protein bodies in the horny endosperm are larger and more numerous than in the floury endosperm (Wolf et al 1967). Starch granules are compacted and polygonal in the horny endosperm, but they are spherical and have space between them in the floury endosperm (Robutti et al 1974). Examination by scanning electron microscopy has revealed the presence of abundant, clinging protein matrix on starch granules in the horny endosperm (Christianson 1970).

Characteristics of horny and floury endosperm suggest that there may be a fundamental difference between their respective cells. Different cell lineages or different stages of differentiation could be present, with more highly differentiated cells occurring in the horny endosperm. Alternatively, hardness could be due, in the simplest case, to the presence of a "hardness protein" or to the absence of a "softness protein." A starch granule protein has been associated with endosperm softness in wheat (Greenwell and Schofield 1986).

To characterize the composition of zeins from endosperm regions that differed in texture, we hand-dissected individual maize kernels to obtain horny and floury endosperm fractions for comparison. Results were related to the model of protein body development proposed by Lending and Larkins (1989).

MATERIALS AND METHODS

Maize Samples

Nine maize samples were analyzed. Inbred B57, supplied by C. M. Wilson, National Center for Agricultural Utilization Research, was grown at the University of Illinois, Urbana, in 1986. Inbred F2, a French flint, was supplied by R. R. Bergquist, Pfister Hybrid Corn Company, El Paso, IL. Hybrid B57 × B68, supplied by C. M. Wilson, was grown at Peoria, IL, in 1988. Hybrids B73 × Mo17, Mo17 × B73, B73 × LH123, and B73 × GC88, supplied by R. R. Bergquist, were grown at El Paso, IL, in 1986. Double cross hybrid 966, supplied by M. W. Lehman, Cornnuts Inc., Oakland, CA, was grown at two different locations (Thorne and Ortega) in Salinas, CA, in 1989. Hybrid 966 has a Cuzco component and is a floury type.

Protein Extraction

Individual kernels were hand-dissected, and portions of endosperm differing in floury and horny texture were separated into their respective fractions for zein protein analysis. Kernels were soaked in distilled water for 5 min, and pericarp and germ were removed. Kernels were allowed to dry overnight. The floury portion was drilled out with a Dremel Moto-Tool (Emerson Electric Company, Racine, IL). The horny portion was ground (approximately 3 min per kernel) in a WIG-L-BUG grinder (Crescent Dental Manufacturing Company, Lyons, IL). Both fractions were passed through a 250-μm-mesh screen. Fractions from 10 to 100 kernels were pooled for analysis. Hard and soft endosperm fractions were weighed to determine percent hard endosperm. Alcohol-soluble proteins were extracted (in duplicate) with 70% (v/v) ethanol and 5% (v/v) 2-mercaptoethanol plus 0.5% (w/v) sodium acetate by shaking on a Buchler vortex-evaporator (Fort Lee, NJ) for 2 hr at room temperature. Endosperm fractions were extracted in 1.5-ml polypropylene tubes, using 50 mg of sample per 250 μl of alcohol solution.

Electrophoresis

After centrifugation, supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A modification of the Laemmli procedure (Fling and Gregerson 1986) was used in which extracts were mixed with an equal volume of sample buffer. Molecular mass standards (Bio-Rad, Richmond, CA) used were phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

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Reversed-Phase High-Performance Liquid Chromatography

Samples also were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) (Paulis and Bietz 1986). Alcohol-soluble proteins were diluted 1:10 with 55% (v/v) isopropanol plus 5% (v/v) 2-mercaptoethanol and separated on a Vydac (Hesperia, CA) reversed-phase (C_{18}) column (25 cm × 4.6 mm, 5-μm particle size, 300-Å pore size). For chromatography, we used solvents A (15% [v/v] acetonitrile [CH_3CN] + 0.1% [v/v] trifluoroacetic acid) and B (80% [v/v] CH_3CN + 0.1% [v/v] trifluoroacetic acid). The nonlinear 60-min gradient began at 25% CH_3CN. The CH_3CN concentration then increased at 1%/min for 25 min, followed by 0.2%/min for 30 min and 1.68%/min for 5 min (to 64.4% CH_3CN). Finally, the column was eluted isocratically at 64.4% CH_3CN for 5 min. The column was operated at 55°C at 1 ml/min. Twenty-microliter samples were injected using a Waters (Milford, MA) WISP 710B automatic sample injector and a Spectra-Physics (San Jose, CA) SP8700 solvent delivery system. Absorbance was monitored at 210 nm (absorbance range = 0.2) with a Beckman (Fullerton, CA) model 165 detector. Data were stored and processed on a ModComp (Ft. Lauderdale, FL) computer system. Analysis of variance and subsequent mean comparison tests were performed using the Student-Newman-Keuls procedure (CoStat Software, Berkeley, CA).

RESULTS AND DISCUSSION

RP-HPLC chromatograms (Fig. 1) of alcohol-soluble endosperm proteins from F2, a French maize flint inbred, illustrate the nomenclature used here for alcohol-soluble maize storage proteins. Peaks (fractions) are labeled 1–4 (Paulis and Bietz 1986). Proteins that eluted last were the most hydrophobic. Numbers in parentheses represent apparent molecular masses, based on SDS-PAGE (Larkins et al 1989, Thompson and Larkins 1989). Apparent molecular masses revealed by SDS-PAGE and actual molecular masses obtained from sequence analysis sometimes differed considerably. The 27-kDa γ-zein can appear as 27–31 kDa by SDS-PAGE (Wilson 1991). Data were plotted as A_{210} to show the amount of each type of alcohol-soluble protein on the basis of constant endosperm weight (value at A_{210} divided by weight). Approximately 70% of zeins in normal maize lines were α-zeins (19 and 22 kDa), encoded by members of multigene families on different chromosomes (Heidecker and Messing 1986, Rubenstein and Geraghty 1986).

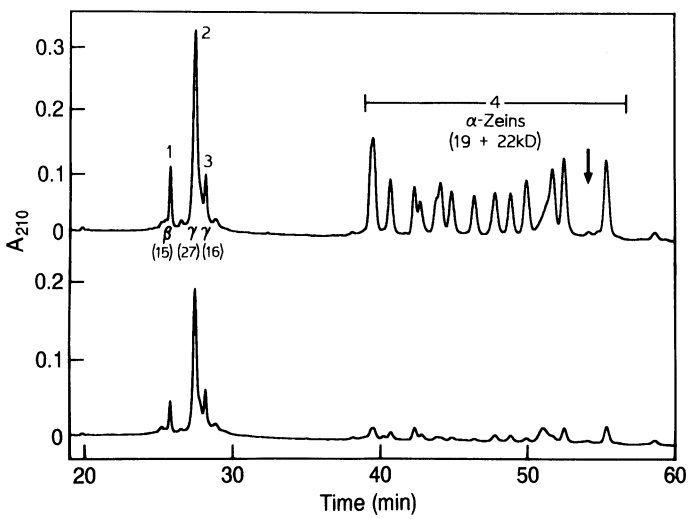

Fig. 1. Reversed-phase high-performance liquid chromatography analyses of alcohol-soluble proteins from hard (top) and soft (bottom) endosperm from F2, a French flint inbred. Total zeins were extracted with 70% ethanol (v/v) containing 5% (v/v) 2-mercaptoethanol and 0.5% (w/v) sodium acetate. Zeins were analyzed using a C_{18} column and a nonlinear 25–64.4% acetonitrile gradient for 60 min. Peaks and peak areas are labeled 1–4 according to Paulis and Bietz (1986). Numbers in parentheses indicate apparent molecular masses, based on SDS-PAGE. The Greek letters α, β, and γ refer to genetic classes, based on sequence homology. An arrow shows the elution position of δ-zein.

Proteins in peaks (fractions) 1–4 were identified by preparative RP-HPLC, followed by SDS-PAGE. Peak 1 contained β-zein (15 kDa). γ-Zeins (27 and 16 kDa) were in peaks 2 and 3, respectively. Multiple peak area 4 contained α-zeins (19 and 22 kDa) and δ-zein (10 kDa) (Kirihara et al 1988, Wilson 1991).

Relative peak percentages and areas of alcohol-soluble proteins from hard and soft endosperm fractions of eight genotypes are presented in Table I. The hard endosperm fraction represented an average of 72% of the total endosperm from all samples except F2, which had 92% hard endosperm (Table II). Differences in total alcohol-soluble protein content of hard- and soft-textured endosperm fractions occurred in flinty (F2) and floury (hybrid 966) genotypes, as well as in cultivars having dent kernels (B57 and other hybrids). Growing conditions in California resulted in hybrid 966 having an increased amount of hard endosperm, relative to the same hybrid grown in Illinois and Ohio (M. A. Dombrink-Kurtzman, *unpublished data*). In each genotype, soft endosperm contained less zein, specifically the many α-zeins in fraction 4, than did hard endosperm. Three main differences in alcohol-soluble proteins occurred between hard and soft endosperm fractions of all inbreds and hybrids analyzed: 1) hard endosperm had more alcohol-soluble proteins (total area) than did soft endosperm; 2) the relative area of fraction 4 (α-zeins) averaged 3.3 times more in hard than in soft endosperm; and 3) the relative percentage of fraction 2 (27-kDa γ-zein) in soft endosperm was approximately twice that in hard endosperm. The

TABLE I
Relative Percentages and Areas of Alcohol-Soluble Endosperm Proteins Determined by Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

		RP-HPLC Fractions[a]				
Genotype	Texture	1	2	3	4	Total Area[b]
B57	Hard	4.9	6.2	7.9	81.0	2,971
	Soft	5.8	17.5	11.0	65.7	714
F2	Hard	3.0	17.3	3.4	76.3	2,739
	Soft	6.0	42.9	10.7	40.4	720
B73 × Mo17	Hard	4.8	13.0	6.3	75.9	1,907
	Soft	4.0	22.9	8.9	64.2	943
Mo17 × B73	Hard	5.0	14.2	6.7	74.0	1,789
	Soft	4.4	23.9	9.5	62.3	917
B73 × LH123	Hard	5.3	8.6	5.6	80.5	2,365
	Soft	4.8	16.6	9.4	69.3	987
B73 × GC88	Hard	6.2	12.2	7.1	74.6	2,425
	Soft	6.2	23.0	11.2	59.6	860
B57 × B68	Hard	4.5	7.6	4.2	83.8	3,148
	Soft	3.6	10.0	5.4	81.1	1,996
966 (Ortega)	Hard	3.9	8.8	8.2	79.1	2,683
	Soft	3.1	12.0	12.8	72.1	1,366
966 (Thorne)	Hard	4.8	13.5	7.6	74.1	2,107
	Soft	3.8	20.5	12.2	63.6	1,005

[a] Relative percentage; average of duplicate extracts.
[b] Total area is the sum of areas of fractions 1–4 (areas not shown). Each figure resulted from the same size sample, therefore, values are proportional to amount of alcohol-soluble protein present.

TABLE II
Percentages of Hard and Soft Maize Endosperm

	Texture	
Genotype	Hard	Soft
B57	60	40
F2	92	8
B73 × Mo17	76	24
Mo17 × B73	70	30
B73 × LH123	80	20
B73 × GC88	71	29
B57 × B68	78	22
966 (Ortega)	69	31
966 (Thorne)	69	31

increased amount of α-zein in hard endosperm was responsible for the greater amount of total alcohol-soluble proteins present in hard endosperm, compared with soft endosperm. Actual amounts of γ-zein were similar in hard and soft endosperm, but γ-zein was present in higher proportion in soft endosperm because of the low level of α-zein. Analysis of variance showed that hard and soft endosperm fractions differed significantly in percentages of fraction 2 (27-kDa γ-zein), fraction 3 (16-kDa γ-zein), and fraction 4 (α-zein) and in total amounts of alcohol-soluble proteins at $P < 0.05$, $P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively.

RP-HPLC results for hard and soft endosperm fractions (Fig. 2A and B, respectively) from hybrid B73 × GC88 and (Fig. 2C and D, respectively) from hybrid 966 (Thorne) show typical distributions of alcohol-soluble proteins (as in Table I). Peak 1 actually contained two major components in most hybrids examined. This correlated with different RP-HPLC retention times reported for 11 inbreds by Wilson (1991). In each inbred, β-zein occurred in peak 1 as one of two possibly allelic variants, differing slightly in elution time and, as analyzed by SDS-PAGE, in apparent molecular mass. Comparison of RP-HPLC chromatograms of reciprocal crosses (hybrids B73 × Mo17 and Mo17 × B73) (Fig. 3) shows similar profiles of alcohol-soluble proteins, regardless of which inbred was the female parent. Because a greater amount of hard endosperm was present in hybrid B73 × Mo17 than in hybrid Mo17 × B73 kernels, whole-kernel analysis of these two hybrids would show a higher content of alcohol-soluble proteins from hybrid B73 × Mo17 than from hybrid Mo17 × B73, possibly related to a dosage effect because endosperm is triploid.

SDS-PAGE analyses of alcohol-soluble proteins from hard and soft endosperm fractions of several hybrids are presented in Figure 4. The gel was purposely overloaded to reveal whether any proteins besides α-, β-, γ-, and δ-zeins were present. Minor bands in the 42.7-kDa region have been observed in overloaded gels (Paulis and Bietz 1986) and probably represent incomplete reduction of β- and γ-zeins. Gels containing less protein also were run. Proteins were extracted from equivalent amounts of endosperm. Samples from soft endosperm contained proportionally more 27-kDa γ-zein (peak 2) than did corresponding samples from hard endo-

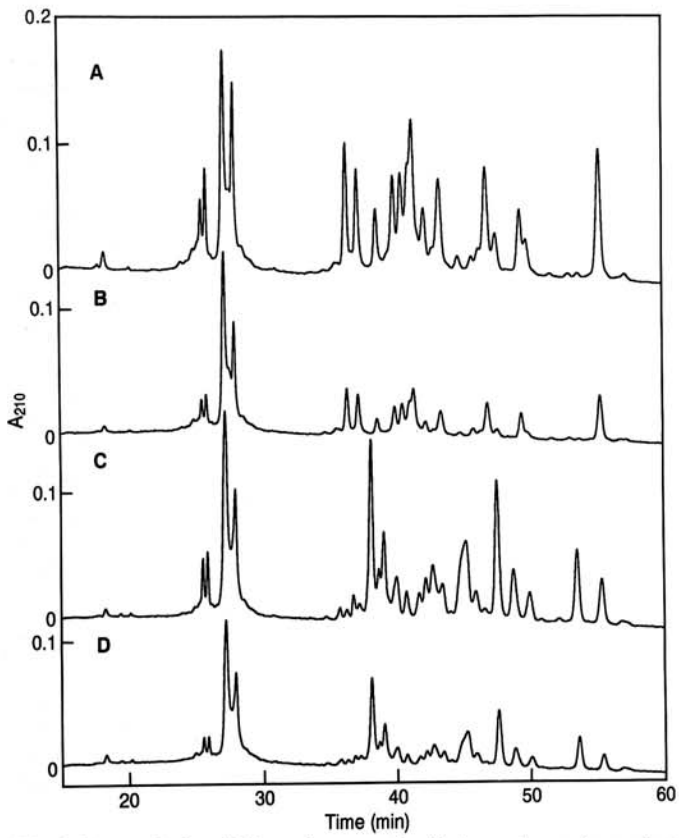

Fig. 2. Reversed-phase high-performance liquid chromatography analyses of alcohol-soluble proteins from hard **(A)** and soft **(B)** endosperm of maize hybrid B73 × GC88 and from hard **(C)** and soft **(D)** endosperm of maize hybrid 966 (Thorne). Total zeins were extracted with 70% ethanol (v/v) containing 5% (v/v) 2-mercaptoethanol and 0.5% (w/v) sodium acetate. Zeins were analyzed using a C_{18} column and a nonlinear 25–64.4% acetonitrile gradient for 60 min. Peaks and peak areas are labeled 1–4 according to Paulis and Bietz (1986).

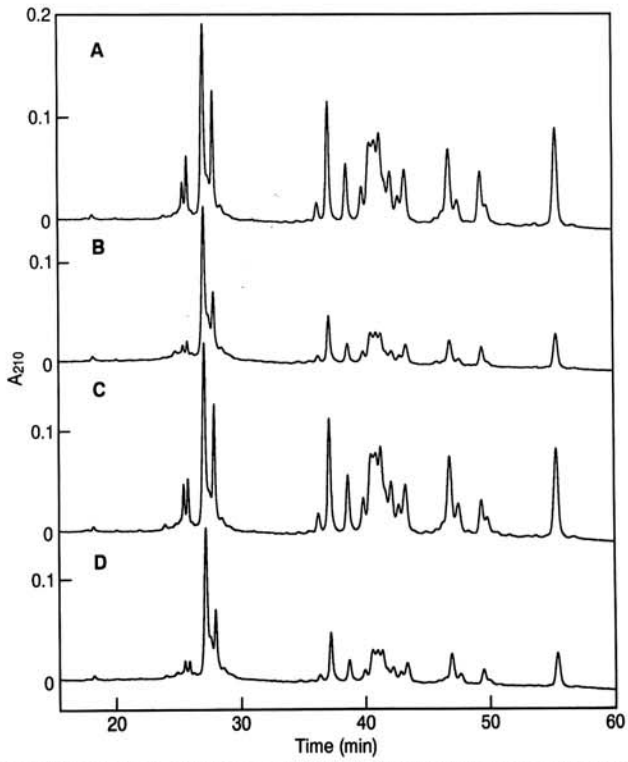

Fig. 3. Reversed-phase high-performance liquid chromatography analyses of alcohol-soluble proteins from hard **(A)** and soft **(B)** endosperm of maize hybrid B73 × Mo17 and from hard **(C)** and soft **(D)** endosperm of maize hybrid Mo17 × B73. Total zeins were extracted with 70% ethanol (v/v) containing 5% (v/v) 2-mercaptoethanol and 0.5% (w/v) sodium acetate. Zeins were analyzed using a C_{18} column and a nonlinear 25–64.4% acetonitrile gradient for 60 min. Peaks and peak areas are labeled 1–4 according to Paulis and Bietz (1986).

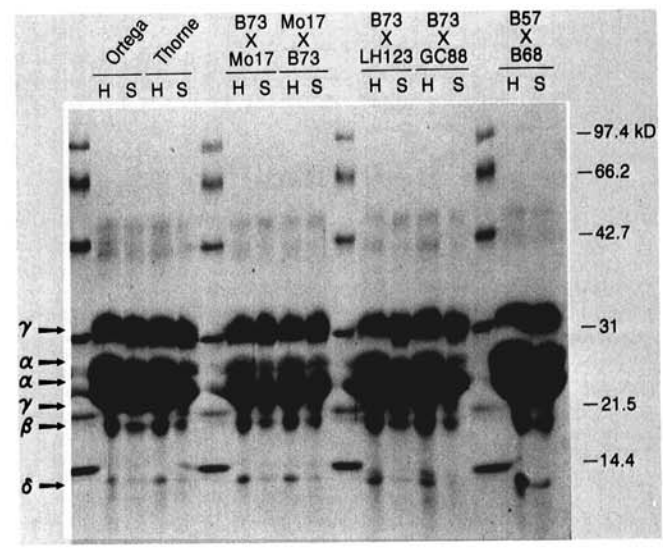

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses of alcohol-soluble proteins from hard (H) and soft (S) endosperm from maize hybrids 966 (Ortega), 966 (Thorne), B73 × Mo17, Mo17 × B73, B73 × LH123, B73 × GC88, and B57 × B68. Genetic classes of proteins are indicated on the left; apparent molecular masses are indicated on the right. Molecular mass standards are in lanes 1, 6, 11, and 16.

sperm. In contrast, hard endosperm contained more α- and δ-zeins (19/22 and 10 kDa, respectively) than did soft endosperm. Relative amounts of the 10-kDa δ-zein, a minor component in fraction 4, are shown in Figure 4.

Examination of zeins did not reveal an alcohol-soluble protein present exclusively in hard or soft endosperm. A salt-soluble 50-kDa protein has been observed only in the soft endosperm of quality protein maize (QPM) populations (Dombrink-Kurtzman and Wilson 1992). Wallace et al (1990), Geetha et al (1991), and Paiva et al (1991) have described increased endosperm hardness in the modified *o2* genotype (QPM) associated with an increase in γ-zein content. Their results for QPM and the results described here for wild type genotypes are not in contradiction. They both represent increased protein synthesis, resulting in a harder endosperm. Because the mutant *o2* genotype does not produce functional *opaque-2* protein, a transcriptional activator of α-zein synthesis (Schmidt et al 1990), increased protein synthesis in QPM lines will not yield an increase in α-zeins. Paiva et al (1991) reported that γ-zein appeared as the major zein in the soft region of the QPM endosperm.

When results from RP-HPLC and SDS-PAGE were compared to the model of protein body development proposed by Lending and Larkins (1989), certain interpretations could be made. First, soft endosperm contained "immature" protein bodies, which have less α-zeins. Second, hard endosperm contained mature protein bodies (with increased amounts of α-zeins). Third, distribution of various types of zeins did not appear to be uniform throughout the maize kernel. Fourth, protein composition of protein bodies may be correlated with the texture of the endosperm fraction from which the sample was obtained. These results emphasize that considerable care is necessary, when analyzing single maize kernels or when sampling a portion of endosperm, to note the texture of the kernel from which the sample was taken, as well as relative amounts of hard and soft endosperm fractions within the kernel.

CONCLUSIONS

Hard and soft endosperm fractions from a Corn Belt inbred and hybrids, as well as from a French flint inbred and maize with a Cuzco component, differed in their composition of alcohol-soluble proteins. More α-zeins occurred in hard than in soft endosperm fractions. Within individual kernels, hard endosperm also contained more total alcohol-soluble proteins than did soft endosperm fractions. In contrast, soft endosperm contained more 27-kDa γ-zein on a percentage basis than did hard endosperm of the same genotype. These differences in protein composition suggest that actual composition of protein bodies in hard and soft maize endosperm fractions may be correlated with endosperm texture in normal maize lines.

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