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Estimation of Protein Quality and Quantity in Corn (*Zea mays* L.) by Assaying Protein in Two Solubility Fractions

Asim Esen

The protein from maize seeds can be recovered in two solubility fractions. Fraction 1 includes zeins (low quality proteins), extracted with 60% *tert*-butyl alcohol, and fraction 2, called nonzein (high quality proteins), includes albumins, globulins, glutelins, and others, extracted with a high pH buffer containing an ionic detergent (sodium dodecyl sulfate) and a reducing agent (2-mercaptoethanol). Protein content of the two fractions, determined by a simple Coomassie Blue R-250 dye-binding method, using comparable proteins as standards, gives an estimate of protein quantity of the sample. The zein as percent of total extractable protein or the ratio of zein to nonzein can be used to estimate the protein quality. The method offers promise as a mass-screening tool to identify the maize germ plasm with high protein and/or high quality protein.

For the improvement of protein quality and quantity in major food crops (e.g., cereals and legumes), the plant breeder needs simple, rapid, inexpensive, and accurate screening techniques to identify the germ plasm with high quality and quantity protein. Such techniques are also needed to monitor progress in a breeding program. The discovery of the *opaque-2* maize, which has almost twice the lysine and tryptophan (major limiting essential amino acids in maize) content as compared to normal maize, by Mertz et al. (1964), was the first breakthrough in the search for high quality protein cereals. Subsequently, other maize mutants with high quality protein were found (Misra et al., 1972). Similar mutants were discovered in barley (Munck et al., 1971) and sorghum (Singh and Axtell, 1973). Unfortunately, no such mutant has yet been found in wheat.

Conventional chemical methods for evaluating protein quality (e.g., amino acid analysis) and quantity (e.g., Kjeldahl nitrogen assay) are either time consuming or require sophisticated and expensive instrumentation. Thus, the need for simple and rapid techniques applicable to mass screening has long been recognized. In the case of maize, the high-lysine trait is associated with decreased zein (prolamines) and increased albumin, globulin, glutelin (Misra et al., 1972), and free amino acid levels (Mertz et al., 1974). Consequently, indirect methods based on zein (Paulis et al., 1974; Dalby, 1974; Esen, 1980) and free amino acid determinations (Mertz et al., 1974) have been developed to screen for protein quality. However, no simple and rapid test is available to screen for protein quantity. This report presents data on the application of a protein determination method, recently developed in our laboratory (Esen, 1978), to estimate protein quality and quantity in maize.

MATERIALS AND METHODS

The whole kernel meals used for protein extraction were from the normal (+) and *opaque-2* (o_2) versions of six inbred lines, namely B37, Mo17, Oh43, Mo2OW, W64A, and Mo2RF. The modified *opaque-2* ($modo_2$) version of the inbred Mo2RF was also available and included in this study.

Protein Extraction. Proteins from all of the 13 samples were separated by differential solubility into two fractions zein (fraction 1) and nonzein (fraction 2). A detailed description of zein extraction was described elsewhere (Esen, 1980) and thus only a brief account will be given here.

For zein extraction, 125 mg of meal was weighed and placed in 15 × 100 mm culture tubes (with screw caps) in duplicate. The meal was suspended in 6.25 mL of 60% *tert*-butyl alcohol (C_4H_9OH), the solvent-to-meal ratio (v/w) being 50:1. Tubes, screw caps tightened, were agitated on a rotary shaker at 150 rpm for 30 min, then transferred in a rack to a waterbath and heated at 70 °C for 15 min. Centrifugation was carried out in a table top centrifuge at 1000*g* for 10 min. The supernatants from duplicates of each sample were pooled; this constituted the zein fraction (fraction 1). The pellet was resuspended in 12.5 mL of 0.1 M sodium borate buffer (pH 10) containing 1% (w/v) sodium dodecyl sulfate ($NaDodSO_4$) and 1% (v/v) 2-mercaptoethanol in order to extract the nonzein fraction (fraction 2). In this case, the solvent-to-meal ratio was 100:1, based on the initial weight of the meal, not that of the pellet. The agitation, heating, and centrifugation schedule used for nonzein extraction was the same as that used for zein. The supernatants from duplicates of each sample were pooled; this constituted the nonzein fraction (fraction 2). Both zein and nonzein fractions were stored in 50-mL Erlenmeyer flasks at room temperature prior to analysis of their protein contents. Routinely, analysis was performed either on the day of extraction or the next day.

In a separate experiment, both zein and nonzein extractions were performed twice in order to check the efficiency of single extraction in solubilizing and recovering proteins belonging to each solubility fraction. Following the first zein extraction, the pellet was resuspended in 6.25 mL of 60% C_4H_9OH for a few seconds and immediately centrifuged, and the supernatant was discarded. The

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Table I. Zein and Nonzein Content of Various *Opaque-2* and Normal Corn Inbreds Determined by Coomassie Brilliant Blue R-250 Dye-binding[a] and Micro-Kjeldahl[b] Methods

	percent protein (mg/100 mg of whole kernel meal ± SD)						
	zein[c]		nonzein		total extractable (zein + nonzein)		ground meal
pedigree	dye-binding	Kjeldahl	dye-binding	Kjeldahl	dye-binding	Kjeldahl	Kjeldahl
B37 +/+	6.28 ± 0.15	5.82 ± 0.18	5.33 ± 0.15	7.22 ± 0.07	11.6	13.0	11.5 ± 0.06
B37 o_2	3.68 ± 0.21	3.35 ± 0.03	7.90 ± 0.10	8.67 ± 0.36	11.6	12.0	11.7 ± 0.03
Mo17 +/+	6.77 ± 0.33	5.76 ± 0.04	5.33 ± 0.24	6.48 ± 0.13	12.1	12.2	11.9 ± 0.03
Mo17 o_2	3.73 ± 0.15	4.36 ± 0.09	7.33 ± 0.21	7.97 ± 0.13	11.1	12.3	12.0 ± 0.03
Oh43 +/+	6.26 ± 0.06	5.32 ± 0.03	5.27 ± 0.20	6.54 ± 0.03	11.5	11.9	11.8 ± 0.12
Oh43 o_2	3.00 ± 0.18	2.34 ± 0.03	7.03 ± 0.22	7.88 ± 0.13	10.0	10.2	10.5 ± 0.14
Mo2OW +/+	7.59 ± 0.32	5.29 ± 0.07	5.30 ± 0.10	6.50 ± 0.09	12.9	11.8	11.6 ± 0.17
Mo2OW o_2	3.66 ± 0.12	2.35 ± 0.04	8.67 ± 0.45	8.73 ± 0.03	12.3	11.1	11.4 ± 0.03
W64A +/+	9.13 ± 0.17	6.71 ± 0.13	6.37 ± 0.40	7.53 ± 0.13	15.5	14.2	14.3 ± 0.12
W64A o_2	3.39 ± 0.20	3.92 ± 0.04	10.20 ± 0.48	9.85 ± 0.07	13.6	13.8	14.0 ± 0.00
Mo2RF +/+	7.53 ± 0.12	6.31 ± 0.02	4.87 ± 0.26	5.82 ± 0.06	12.4	12.1	12.3 ± 0.10
Mo2RF o_2	4.10 ± 0.19	4.72 ± 0.04	7.97 ± 0.40	8.89 ± 0.06	12.1	13.6	13.5 ± 0.10
Mo2RF modo_2	4.21 ± 0.29	4.75 ± 0.03	7.97 ± 0.42	8.91 ± 0.03	12.2	13.7	13.8 ± 0.10

[a] Dye-binding values are the mean of three assays. [b] Micro-Kjeldahl values are the mean of two assays. [c] These zein data also appear in another report by Esen (1980).

purpose of this wash was to remove zein left from the first extraction in the pellet matrix and inner surface of the culture tube so that it would not contaminate the second extract. Similarly, a brief wash with the solvent preceded the second extraction in the case of nonzein. The schedules for the second zein and nonzein extractions were the same as those used for the first ones.

Preparation of Zein and Nonzein Standards. One hundred milligrams of meal from each of 13 samples was mixed together in a 500-mL flask to yield 1.3 g of pooled meal. Zein and nonzein fractions were extracted from this pooled meal. The solvent-to-meal ratio (v/w) was 20:1. Subsequent to zein extraction the pellet was extracted four more times with 60% C_4H_9OH to insure complete release of zein so that it would not contaminate the nonzein fraction to be extracted next. Nitrogen contents of the zein and nonzein fractions were determined in duplicate according to micro-Kjeldahl procedure approved by the American Association of Cereal Chemists (1962), and nitrogen was converted to protein by multiplying by the factor of 6.25. These zein and nonzein fractions were used as protein standards for the assay of zein and nonzein by dye-binding. For this, the zein fraction was diluted so as to obtain standard solutions ranging in concentration from 0.4 to 1.89 mg/mL, while the range in nonzein solutions was 0.5 to 1.35 mg/mL.

Zein and Nonzein Determination. Zein and nonzein protein from each sample was determined according to Coomassie Blue R-250 dye-binding method developed by the author (Esen, 1978, 1980). The protein standards were zein and nonzein solutions with known nitrogen content, prepared as described above. Ten-microliter aliquots of the unknown (zein and nonzein fractions from + and o_2 genotypes) and appropriate standards were spotted in triplicate on rectangular (17 × 22 cm) sheets of Whatman No. 1 chromatography paper and stained in a glass tray with 0.1% Coomassie Brilliant Blue R-250 solution for 15 min. The nonspecifically bound dye was removed by destaining with water for 10 min. The stained protein spots were cut with a cork borer, and the dye was eluted with 1% $NaDodSO_4$ solution. The absorbance of the dye in the eluate was read at 595 nm. Zein and nonzein contents of unknown were read off from the standard curve and expressed as mg/100 mg of whole kernel meal.

Two 5-mL aliquots from zein and nonzein fractions of each sample were used to determine their nitrogen contents by the micro-Kjeldahl procedure. In addition, the nitrogen content of whole kernel meal from each sample was determined in two 100-mg samples. Nitrogen values were multiplied by 6.25 for conversion to protein.

Table II. Percent Zein (as Percent of Protein), Zein-to-Nonzein Ratio, and Lysine Content in Various *Opaque-2* and Normal Corn Inbreds

	zein as percent of total extractable protein		zein-to-nonzein ratio		Lys as % of protein[a]
pedigree	dye-binding	Kjeldahl	dye-binding	Kjeldahl	
B37 +/+	54.1	44.8	1.18	0.81	2.96
B37 o_2	31.7	27.9	0.47	0.39	4.27
Mo17 +/+	56.0	47.2	1.27	0.89	2.94
Mo17 o_2	33.6	35.4	0.51	0.55	3.98
Oh43 +/+	54.4	44.7	1.19	0.81	2.91
Oh43 o_2	30.0	22.9	0.43	0.30	4.31
Mo2OW +/+	58.8	44.8	1.43	0.81	2.76
Mo2OW o_2	29.8	21.2	0.42	0.27	4.50
W64A +/+	58.9	47.3	1.43	0.89	2.73
W64A o_2	24.9	28.4	0.33	0.40	4.71
Mo2RF +/+	60.7	52.1	1.55	1.08	2.74
Mo2RF o_2	33.9	34.7	0.51	0.53	3.64
Mo2RF modo_2	34.5	34.7	0.53	0.53	3.69

[a] Lysine data were kindly provided by M. S. Zuber.

RESULTS AND DISCUSSION

Zein and Nonzein Content of Normal and *Opaque-2* Inbreds. The data showed that normal inbreds consistently had higher zein content than their *opaque-2* counterparts (Table I). This was not unexpected in view of similar results obtained by other investigators (Misra et al., 1972, 1975). The Coomassie Brilliant Blue R-250 dye-binding and micro-Kjeldahl methods in general gave similar zein values. Both methods would enable one to distinguish + and o_2 genotypes from each other on the basis of their zein contents since + and o_2 zein values did not overlap. However, there were differences among o_2 and + versions of different inbreds. The zein contents of o_2 mutants as percentage of total extractable protein varied from 24.9 to 33.9%, while those of their + counterparts varied from 54.1 to 60.7% when protein was determined by the dye-binding method (Table II). The corresponding

ranges were 21.2–35.4% for o_2 mutants and 44.7–52.1% for normals when protein was determined by the micro-Kjeldahl method. These values, especially in the case of the o_2 mutants, are somewhat higher than those reported by others (Misra et al., 1972; Dalby, 1974). The discrepancy probably results from increased extractability of zeins in the presence of a large solvent-to-meal ratio and heating during extraction. For example, it was found that the Landry–Moureaux procedure (Landry and Moreaux, 1970), which uses a 10:1 solvent-to-meal ratio (v/w) and three successive extractions with 70% isopropyl alcohol, does not release zeins completely (Esen, unpublished). When these three successive extractions were followed by extraction with 60% C_4H_9OH at 70 °C, additional zein was released, which amounted to 22% of the protein released by the first three extractions. The electrophoretic profiles of zeins (not shown) from all four extracts were similar, which suggested that the heat-released zein is the same as that released by the first three successive extractions.

When nonzein contents were compared, the trend was completely reversed; + versions of all inbreds contained lower nonzein than their o_2 counterparts (Table I). The nonzein fractions (fraction 2) includes albumins, globulins, and glutelins (including zein-like proteins), which was verified by comparing the electrophoretic profiles of the Landry–Moureaux fractions with those of nonzein. Thus, the data indicate that decrease in the zein fraction is consistently accompanied by increase in the nonzein fraction. This is in agreement with the findings of Misra et al. (1972) who found that decrease in the zein content of *opaque-2* mutants resulted in an increase in albumins, globulins, and glutelins. Furthermore, nonzein protein values from o_2 inbreds did not overlap with those from normals, that is, the o_2 mutant with the lowest nonzein content had higher nonzein protein than the + inbred with the highest amount of nonzein protein. With one exception ($W64Ao_2$), the amount of nonzein protein estimated by micro-Kjeldahl method was somewhat higher than that estimated by the dye-binding method. This may be due to nonprotein nitrogen that nonzein protein contains, which is not detected by dye-binding.

Efficiency of Extraction Procedure. The two-step sequential extraction procedure employed to separate corn proteins into two critical fractions, lysine-poor zeins and lysine-rich nonzeins, was found to release quantitatively nearly all of the protein present in the sample. Two consecutive extractions with the same solvent showed that the first extraction released 92–98% of extractable zein (mean 95.6%) and 96–98% of extractable nonzein (mean 97.2%) based on results from the dye-binding assay. In fact, often the spot of the second extract after staining could not be visually detected on paper. Thus, extraction with zein and nonzein solvents, once each, suffices; repeating extraction with the same solvent is not warranted unless one is interested in estimations of the quantity of extractable protein with utmost precision. The comparisons of total extractable protein values (zein + nonzein) with those determined in ground meal showed considerable agreement (Table I). Total extractable protein values estimated by dye-binding were slightly lower than those in the ground meal (micro-Kjeldahl) in the case of o_2 mutants but higher, with one exception, in the case of normals. This may be reflecting differences between o_2 and + inbreds with respect to free amino acid content since o_2 mutants contain three–four times more free amino acids than normals (Mertz et al., 1974). Dye-binding does not detect nonprotein nitrogen (e.g., free amino acids, small peptides); therefore, it theoretically should give lower estimates of protein in o_2 mutants and higher estimates of protein in normals than the Kjeldahl method. This would be expected if the extract used as the protein standard had an intermediate free amino acid content. As mentioned above, the protein standard for dye-binding used in this study came from a mixture of meals from o_2 and normal inbreds. Results in Table I appear to be in support of this explanation.

Table III. Correlations between Zein and Nonzein Values Determined by Dye-binding and Micro-Kjeldahl Methods and between Two Quality Indices and Lysine

variables	correlation coeff (r)
zein (KP)[a] and zein (DBP)[b]	0.87[d]
nonzein (KP) and nonzein (DBP)	0.97[d]
zein (KP) as % of total extractable protein (KP) and Lys[c]	−0.95[d]
zein (DBP) as % of total extractable protein (DBP) and Lys	−0.97[d]
zein (KP)/nonzein (KP) ratio and Lys	−0.94[d]
zein (DBP)/nonzein (DBP) ratio and Lys	−0.95[d]

[a] KP, Kjeldahl protein. [b] DBP, dye-binding protein. [c] Lysine as percent of protein. [d] Significant at $P = 0.01$.

Correlation of Dye-binding Protein with Kjeldahl Protein. The Coomassie Blue R-250 dye-binding method yielded protein values that were highly correlated with values obtained by the micro-Kjeldahl method. For example, correlation between the two methods was 0.87 and 0.97 for zein and nonzein, respectively (Table III). However, it should be emphasized that the principle of the two methods is different. The Kjeldahl method measures nitrogen, while the dye-binding method is based on the binding of an anionic dye to basic amino acid residues and the free amino group of the N-terminus residue of proteins. Free amino acids and small peptides do not form insoluble complexes with the dye; they are lost during staining and destaining and consequently not detected. Therefore, any discrepancy between the two methods can be partly attributed to differences in free amino acids, small peptides, proteins rich in basic amino acid residues, and nonprotein and nonamino acid nitrogen contents among different samples. This would hold true even when a standard with known Kjeldahl nitrogen content is used since the standard and unknowns would differ with respect to variables mentioned above. Furthermore, the dye-binding method tends not to be as reproducible as the Kjeldahl method. For example, in this study the coefficient of variation for Kjeldahl protein values varied from 0.02 to 4.2%, while for dye-binding it varied from 1.0 to 6.9%, the major sources of variation being the difficulty of pipetting small sample volumes (5–10 μL) accurately and nonuniform background staining across the paper. Thus, when dye-binding is used to measure protein, a comparable standard is necessary. In addition, allowance should be made for up to ±7% error.

Relationship among Percent Zein, Zein/Nonzein Ratio, and Lysine. The data indicate that the two-step sequential extraction procedure and assay of protein in the two resulting solubility fractions (zein and nonzein) by the Coomassie Blue R-250 dye-binding method can give a reasonably accurate estimate of protein quality and quantity in corn samples (Tables I–III). In this procedure, the sum of protein in the zein and nonzein fractions can be used to estimate the amount of protein in the sample. Results show that such an estimate is in close agreement with the amount of total extractable protein and that determined in whole kernel meal by the micro-Kjeldahl method. Moreover, an estimate of potential protein quality can be made from one of the two indices that are easy to

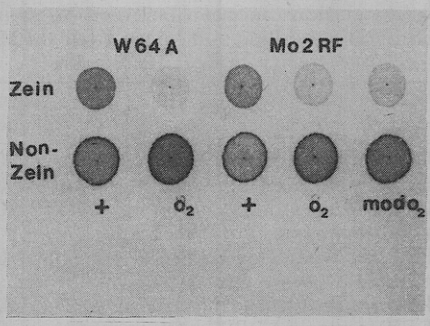

Figure 1. Stained zein and nonzein spots from normal (+) and *opaque-2* (o_2) versions of two corn inbreds, W64A and Mo2RF. In addition, spots from modified *opaque-2* ($modo_2$) version of the inbred Mo2RF is shown. Note that o_2 and $modo_2$ inbreds can be distinguished from their normal counterparts by reduced intensity of their zein and increased intensity of their nonzein spots.

calculate. One of the indices is zein as percent of total extractable protein or zein/zein + nonzein. This index varied from 24.9 to 34.5% in o_2 mutants from 54.1 to 60.7% in + inbreds (Table II). Since there is an inverse relationship between zein and lysine, zein as percent of total extractable protein showed a high negative correlation with lysine ($r = -0.97$) (Table III). When the same index was calculated from zein and nonzein values determined by the micro-Kjeldahl method, the correlation coefficient was slightly lower ($r = -0.95$) than that calculated from dye-binding values (Table III). The other index of protein quality calculated was the zein/nonzein ratio. The index varied from 0.33 to 0.53 in o_2 mutants from 1.18 to 1.55 in normals. Likewise, there was an inverse relationship between this index and lysine content, the correlation coefficient being highly negative ($r = -0.95$). Calculation of the same index from zein and nonzein protein determined by the micro-Kjeldahl yielded essentially the same high negative correlation with lysine content ($r = -0.94$). On the basis of these results, the following generalization can be made: The lower the percent zein and the zein-to-nonzein ratio, the higher the protein quality. It should be noted that both protein quality indices clearly separated o_2 and + genotypes into two distinct classes with no overlapping of values between them.

The method described is suitable for estimation of protein quality and/or quantity simultaneously in corn germ plasm. Preliminary results indicate that it is equally suitable for estimation of protein quality and quantity in barley, sorghum, and wheat (data not shown). It is also applicable to single seed analysis. Because of the small sample requirement (10–20 μL), analysis can be performed on samples weighing as little as 1 mg if they can be weighed accurately and rapidly. Perhaps a sample weight of 5–10 mg would be more realistic in practice. Preliminary studies show that it is possible to carry out the analysis on 5–10 mg of meals from endosperm slices and plant the remainder of the kernel with no adverse effects on germination. This would be very useful for a plant geneticist who is interested in protein quality and quantity status of individual seeds in segregating breeding populations. It should be mentioned that a 5–10-mg sample may not be truly representative of the whole seed and could lead to erroneous conclusions unless segregants differ considerably in protein quality and quantity.

Potential of Dye-Binding in Mass-Screening for Protein Quality and/or Quantity. *Opaque-2* and other high-quality protein mutants can be distinguished from normal corns, without any quantitative assay, by visually inspecting the intensity of their respective zein and nonzein spots after staining, as is evident from Figure 1. Thus, the dye-binding method can serve as a simple, inexpensive and rapid tool in preliminary screening for high quality and/or quantity protein types. In this case it is possible to spot zein and nonzein fractions from up to 200–250 samples on a single sheet of 17 × 22 cm chromatography paper. After staining for 10–15 min and destaining for 5 min, the potential high protein or high quality protein types can be selected on the basis of the staining intensity of their spots judged by visual examination. These selected samples can then be subjected to conventional quantitative analysis (e.g., Kjeldahl nitrogen determination and amino acid analysis) to verify their status more accurately with respect to protein content and quality. In fact, one can even obtain a semiquantitative estimate of the zein and nonzein content of the samples by spotting standards with known protein content on the same paper and comparing visually the staining intensity of spots from unknowns with those of standards.

Additional Remarks. (1) Zein and nonzein solutions were remarkably stable when stored in screw-capped vials at room temperature; virtually the same values were obtained when the same solutions were analyzed 6 months apart. (2) Chromatography paper sheets can be stored after samples are spotted or stained for months or longer. (3) Although the method was found to be free from interference by nonprotein substances and common laboratory reagents (except detergents), it would be desirable to prepare standards in the same solvent as used for unknowns. (4) Commercially available sources of Coomassie Brilliant Blue R-250 considerably differ in their dye contents. A 0.1% solution of the dye made from a batch with sufficiently high dye content is adequate for maximum staining and sensitivity. However, some batches of the same dye purchased from the same or different suppliers had low dye contents. In these instances, staining solutions containing 0.15–0.2% dye were required to obtain the desired staining intensity.

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