A Simple Colorimetric Method for Zein Determination in Corn and Its Potential in Screening for Protein Quality

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ABSTRACT

A method based on the quantitative binding of Coomassie Brilliant Blue R-250 to proteins spotted on Whatman No. 1 chromatography paper was used to determine zein in 60% tertiary butanol extracts from whole corn kernel powders. The normal and *opaque-2* versions of six inbreds could be easily distinguished on the basis of their zein content, *opaque-2* genotypes having a consistently lower percentage of zein than their normal counterparts. The method is specific for protein (zein) and has potential uses as a quantitative and semiquantitiative tool in mass screening of germ plasm sources and single seeds for protein quality.

The alcohol-soluble protein fraction, zein, makes up 40–60% of the protein in the corn kernel. Zein is extremely deficient in lysine and tryptophan and is primarily responsible for the low nutritional quality of corn as a protein source for monogastric animals, including man. Since Mertz et al (1964) discovered the high-lysine corn *opaque-2* (which is low in zein), considerable research effort has been expended to develop hybrids with high quality protein, but problems such as lower grain yields, higher moisture content, chalky and dull appearance, and susceptibility to certain pests have hindered the commercial acceptability of the *opaque-2* hybrids. Dudley et al (1975) showed that some *opaque-2* hybrids could produce yields as good or even better than some commercial dents. Moreover, hard vitreous endosperm types of *opaque-2* corn have recently been developed (Dudley et al 1975, Vasal 1975). Selecting for lysine content without utilizing any of the known mutant genes (o_2,fl_2, etc) is another alternative approach for breeding high quality protein corn (Zuber and Helm 1975). Although improvement of protein quality of corn and other cereals with protein supplements from other sources and with synthetic amino acids is possible, in the long run the improvement by genetic means appears to be the most economical and least complicated approach.

The genetic development of corn hybrids with high quality protein hinges, for the most part, upon the development and use of simple, rapid, and accurate methods for lysine and/or tryptophan determination. Methods that measure lysine directly (eg, automatic amino acid analysis) are time-consuming and require skilled personnel and/or sophisticated and expensive instruments. The dye-binding method of Mossberg (1969) is rather simple but not specific for lysine because it uses azo dye, which binds also to histidine and arginine. Indirect methods based on the positive correlation between lysine and butanol-insoluble nitrogen in corn meal (Fromberg et al 1971) and between lysine and free amino acid (Mertz et al 1974, Misra et al 1975b), and those based on the negative correlation between lysine and zein content (Dalby 1974, Jones et al 1975, Paulis et al 1974) are also available.

This paper reports on a direct zein determination method that is simple, rapid, specific, and reproducible. The method can be used as a quantitative or semiquantitative tool in screening corn germ plasm and breeding materials for protein quality.

MATERIALS AND METHODS

The whole kernel meals used in this study were from the normal and *opaque-2* (o_2) versions of six inbred lines (B37, Mo17, Oh43, Mo20W, W64A, and Mo2RF). A modified *opaque-2* version of one of these lines, Mo2RF, was also included. Whole kernel meals were provided by M. S. Zuber of the University of Missouri, Columbia. Dr. Zuber also provided the lysine data for each sample.

Zein Extraction

Duplicate samples (125 mg) of whole kernel meal from each line were placed in 15-ml pyrex culture tubes with screw-caps. Then 60% tertiary butyl alcohol (6.25 ml) was added to each tube and the screw-cap was tightened; the ratio of solvent volume to sample weight was 50:1. The meal was suspended in the solvent on a Vortex mixer for a few seconds, and then the tubes were placed horizontally on the platform of a rotary shaker. They were agitated at 150 rpm for 30 min, transferred in a test tube rack to a waterbath,

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and heated at 70°C for 15 min. During heating, tubes were individually taken out of the waterbath and vortexed for a few seconds at 5-min intervals. Tubes were then placed in an IEC benchtop centrifuge and spun at 1,000 × *g* for 10 min. The supernatants, which contained zein, from duplicates were pooled and stored in 50-ml Erlenmeyer flasks at room temperature until use.

Preparation of Zein Standard

Whole kernel meal (100 g) from each of 13 samples was weighed and mixed together in a 500-ml flask to yield 1.3 g of pooled meal representing all samples. Zein was extracted from this pooled meal with 26 ml of 60% t-butanol by stirring continuously on a magnetic stir motor for 30 min, then heating at 70°C for 15 min. The ratio of solvent volume to sample weight was 20:1. The content of the flask was transferred to 50-ml centrifuge tubes and spun in a Sorvall RC2-B centrifuge at 12,000 × *g* for 15 min. The supernatant was saved to use as zein standard (stock solution). Nitrogen content of this solution was determined in two 5-ml samples according to the micro-Kjeldahl procedure (AACC), and the factor 6.25 was used to convert nitrogen to protein. The protein (zein) concentration of the stock solution was 2.70 mg/ml. Appropriate dilutions from this solution were made with 60% t-butanol to obtain standard solutions with concentrations ranging from 0.4 to 1.89 mg of zein per milliliter.

Zein Determination

Zein was determined according to a dye-binding assay for protein (Esen 1978). The only modifications were that the absorbance was read at 595 nm for increased sensitivity, and the protein standard was composed of zein solutions with known protein content, prepared as described. Briefly, 10-μl aliquots from the zein extract of each sample were spotted in triplicate with a Hamilton syringe on rectangular sheets (17 × 22 cm) 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, during which the water was changed twice. Along with the unknowns, each sheet also contained spots of standard zein solutions. The stained zein spots, after drying, were cut with a cork borer, and the dye from each spot was eluted with 3 ml of 1% sodium dodecyl sulfate solution in 2-dram specimen vials for 30 min or longer. The absorbance of the dye in the eluate was read at 595 nm. The zein concentrations (in milligrams per milliliter) of the standard solutions were plotted against the mean absorbance of their eluates to obtain the standard curve. The zein content of unknowns (zein extracts from normal and o_2 samples) were read off from the standard curve as milligrams per milliliter, and appropriate calculations were carried out to convert these values to milligrams of zein per 100 mg of whole kernel meal.

Two 5-ml aliquots from the zein solutions of each sample were used to determine nitrogen content, according to the micro-Kjeldahl procedure. In addition, the nitrogen content of whole kernel meal from each sample was determined in 100-mg samples in duplicate. The percent nitrogen values were converted to percent protein by multiplying by the factor 6.25.

TABLE I
Zein Content of Normal and *Opaque-2* Versions of Six Corn Inbreds Determined by Micro-Kjeldahl and Coomassie Billiant Blue Dye-Binding Methods

Pedigree[a]	Zein Content (mg/100 mg of whole kernel meal)		Protein in Whole Kernel Meal[c] (%)	Lysine as Percent of Protein[d]
	Dye-binding[b]	Micro-Kjeldahl[c]		
B37 +/+	6.28	5.82	11.5	2.96
B37 o_2	3.68	3.35	11.7	4.27
Mo17 +/+	6.77	5.76	11.9	2.94
Mo17 o_2	3.73	4.36	12.0	3.98
Oh43 +/+	6.26	5.32	11.8	2.91
Oh43 o_2	3.00	2.34	10.5	4.31
Mo2OW +/+	7.59	5.29	11.6	2.76
Mo2OW o_2	3.66	2.35	11.4	4.50
W64A +/+	9.13	6.71	14.3	2.73
W64A o_2	3.39	3.92	14.0	4.71
Mo2RF +/+	7.53	6.31	12.3	2.74
Mo2RF o_2	4.10	4.72	13.5	3.64
Mo2RF modo_2	4.21	4.75	13.8	3.69

[a] +/+ = normal line, o_2 = *opaque-2* line, modo_2 = modified *opaque-2* line.
[b] Mean of three assays.
[c] Mean of two assays.
[d] Lysine data for all samples were provided by M. S. Zuber, University of Missouri, Columbia.

RESULTS AND DISCUSSION

Sensitivity, Specificity, and Reproducibility of Dye-Binding Assay for Zein

The method was sensitive enough to assay zein in extracts as low as 0.1 mg/ml or 1 μg per spot. The concentrations below 0.05 mg/ml did not produce a visible spot on the paper after staining, and the absorbance of the eluate was virtually the same as that of the blank. However, this poses no serious problem because even zein extracts from corn with the lowest zein content contain more than 0.6 mg of protein per milliliter.

Coomassie Brilliant Blue R-250 dye binds specifically to proteins (Fazekas de St. Groth et al 1963) and is considered to be the most sensitive protein stain. Attempts to stain a variety of nonproteinaceous substances yielded unequivocally negative results (Esen 1978). Although the dye is specific for protein, different amounts of dye bind to the same amount of different proteins.[1] For example, the amount of dye that binds to 1 μg of bovine serum albumin (BSA) is about five times greater than the amount that binds to 1 μg of zein. A study using synthetic polyamino acids showed that the dye binds to the basic amino acid residues, eg lysine, arginine, and histidine.[1] According to the amino acid analysis data reported by Misra et al (1975a), the zein fraction of corn endosperm proteins contains a total of 3.7% basic amino acids. The basic amino acid content of BSA is 16.68%, which is about 4.5 times that of the zein and accounts for the five-fold difference in the amount of dye that binds to equal amounts of zein and BSA.

Triplicate assays of zein extracts from normal and o_2 versions of inbreds yielded highly reproducible results (Table I). The coefficient of variation varied from 1 to 6.9 percent. The major sources of variation were the difficulty in accurately pipetting the small sample volumes (10 μl) and the nonuniformity of the background stain in the paper. The reproducibility was therefore somewhat lower than that obtained by the micro-Kjeldahl method.

Zein Content of Normal Corn Inbreds and Their *Opaque-2* Counterparts

The use of the described method for zein determination in normal and o_2 versions of six inbreds showed that, as expected, normal inbreds contained up to 2.7 times more zein than their o_2 counterparts. Results are summarized in Table I, which also includes zein values determined by the micro-Kjeldahl method for comparison. The magnitude of differences in zein content between normal and o_2 versions was somewhat lower than that reported in the literature. This smaller difference may be due to the increased efficiency of zein extraction, especially from o_2 samples, because of the high solvent volume to sample weight ratio (50:1). Heating during extraction also increased the recovery of zein. In separate experiments, an additional extraction yielded less than 5% of the amount of zein recovered in the first extraction, indicating that the first extraction released nearly all the zein present in the sample.

A comparison of the dye-binding and micro-Kjeldahl methods showed that the former gave consistently higher zein values in extracts from normal inbreds than the latter did (Table I). However, this trend did not hold in extracts from o_2 inbreds, where the dye-binding method yielded lower zein values in four out of

[1] Esen, unpublished.

seven inbreds than did the micro-Kjeldahl method. The source of discrepancy in the values obtained by the two methods is in the basis by which they measure protein. The dye-binding measures protein, whereas the micro-Kjeldahl measures nitrogen and assumes that all the nitrogen measured is contributed by protein. Mertz et al (1974) showed that o_2 corn genotypes contained three to four times more free amino acids than normal genotypes did. Because the zein standard used in this study was extracted from a mixture of meals from seven o_2 and six normal inbreds, it may have contained some free amino acids in addition to zein. Thus, the use as protein standard of a zein solution that contains nonprotein nitrogen (eg free amino acids) would result in overestimation of zein in extracts from normal inbreds and underestimation in those from o_2 inbreds when zein is determined by dye-binding. Nevertheless a high correlation ($r = 0.872$, $P = 0.01$) was found between zein values obtained by the micro-Kjeldahl and the dye-binding methods.

Relationship Between Zein and Lysine

The existence of a high negative correlation between zein and lysine contents has been well-documented in corn (Misra et al 1972, Paulis et al 1974). The same holds true for zein and tryptophan because lysine and tryptophan levels are positively correlated (Hernandez and Bates 1969). Consequently, protein quality of corn is negatively correlated with zein content because zein makes up 50–60 percent of the protein in the corn kernel and is deficient in lysine and tryptophan—the major limiting essential amino acids. The negative relationship between zein and lysine contents is clearly evident from the data in Table I. In all cases, o_2 mutants have lower zein and higher lysine levels than their near-isogenic normal counterparts. Correlation coefficients between zein and lysine are listed in Table II. Whether or not zein was expressed as percent of whole kernel meal or as percent of protein, it showed a high negative correlation (90% or greater) with lysine. Moreover, both dye-binding and micro-Kjeldahl methods yielded virtually the same correlation coefficients. However, expressing zein as percent of total protein produced a slightly higher negative correlation with lysine than did expressing zein as percent of sample weight (Table II). These results indicate that the dye-binding method described in this report can assess the protein quality status of a corn sample with reasonable accuracy and can distinguish mutants with improved protein quality.

Potential Uses in Screening for High-Quality Protein Mutants

Because a high negative correlation exists between zein and lysine content (Misra et al 1972, Paulis et al 1974), a method measuring zein can evaluate the lysine status of a corn sample indirectly. Such a method will be valuable especially in identifying the hard vitreous endosperm types of *opaque-2* corn and other high-lysine mutants phenotypically indistinguishable from normal corn. The turbidimetric method developed by Paulis et al (1974) gives an indirect estimate of lysine and has been used for this purpose. It is not completely specific for zein, however, because alcohol-soluble lipids (Paulis et al 1974) and pigments[2] produce turbidity when 1% NaCl and other aqueous solutions are added to alcohol extracts. Dalby's method (1974) requires time-consuming manipulations. Its modification (Jones et al 1975), utilizing the Lowry assay instead of Kjeldahl nitrogen, does not correlate well with A_{280} readings, suggesting the presence of phenolic substances that interfere with the Lowry assay and may yield unrealistic protein values (Loomis, 1974). In fact, the rapid ninhydrin color test for free amino acids developed by Mertz et al (1974) is more suitable for mass screening than either of the other methods.

The method described in this report offers some unique features and advantages in screening for corn mutants with improved protein quality. 1) It is free from interference by nonzein substances (eg lipids, free amino acids, and phenolic substances) and is specific for protein. 2) It uses zein of known nitrogen content as the protein standard; zein content of an unknown can be directly read from the standard curve. 3) The color intensity of the dye-zein complex is linearly proportional to the zein concentration up to 10 mg/ml (data not shown), and virtually every zein extract can be directly spotted on paper and assayed without dilution. 4) The sample volume required is rather small, 20 μl for duplicate assays, which means that kernel or endosperm sections as small as 1 mg can be used for analysis if weighed accurately. A small sample requirement can be of great value when nondestructive single seed analysis is necessary; a small endosperm part could be removed for analysis and the remainder of the kernel kept for planting. A corn kernel weighs 200–300 mg; a small slice of endosperm (5–10 mg) can be removed from kernel, crushed to a fine powder in a glass mortar, weighed, and used for zein extraction. Single seed analysis in endosperm meals weighing 3 mg was found to be possible by extracting zein in small test tubes. 5) The method is simple and rapid. The larger the number of samples to be analyzed, the less time per sample is required. A sheet of chromatography paper measuring 17 × 22 cm can accommodate 20–30 samples for duplicate assays. 6) The cost of analysis is minimal because the reagents are inexpensive and have long shelf life. For example, the staining solution can be used 10 times or more. The major cost items are labor and a spectrophotometer. 7) The method does not require critical timing of operations except in the staining and destaining steps. 8) One unique feature of the method is that it allows permanent records of stained zein spots to be kept. Samples can be spotted on two separate papers, one for quantitative assays after elution of stained spots, and the other, after staining and drying, to file for future reference.

Semiquantitative Assay and Applications for Mass Screening

Semiquantitative estimation of the amount of zein is possible if varying dilutions of the zein (eg 0.2, 0.4, 0.6 . . . 1.0, 2.0 mg/ml) with known protein content are spotted on the same paper with unknowns and the color intensities of spots from unknowns are compared with those from standards. Such assays can be extremely rapid because up to 250 samples can be spotted with a precalibrated micropipet on the same sheet, stained, destained, and evaluated in an hour or so. Evaluation may be done immediately after destaining while the paper is wet or at a later time. Visual comparison of stained spots with those from the dilution series of the standard can approximate zein concentration of unknowns

TABLE II
Relationships Between Zein and Lysine as Determined by the Micro-Kjeldahl and the Coomassie Brilliant Blue Dye-Binding Methods

Variables	Method of Zein Determination	Correlation Coefficient[a]
Zein (as % of whole kernel meal) and lysine[b]	Micro-Kjeldahl	−0.896
Zein (as % of whole kernel meal) and lysine	Dye-binding	−0.920
Zein (as % of protein in ground sample) and lysine	Micro-Kjeldahl	−0.946
Zein (as % of protein in ground sample) and lysine	Dye-binding	−0.932

[a] $P = 0.01$ in all cases.
[b] Lysine expressed as percent of protein in all cases.

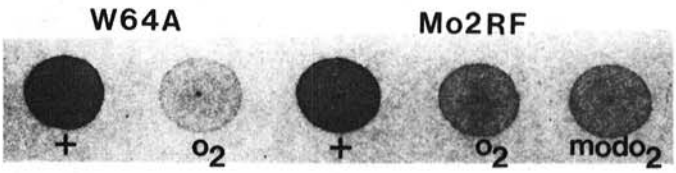

Fig. 1. Stained zein spots from normal (+) and *opaque-2* (o_2) versions of two corn inbreds, W64A and Mo2RF. For comparison, zein spot from modified *opaque-2* (modo_2) version of inbred Mo2RF is also included. Sample volume, 10 μl. The o_2 and modo_2 samples can be easily distinguished from normals by reduced intensity of their stained zein spots.

[2] Esen, unpublished.

with an error of ± 15 percent.

Perhaps the most important feature of the method is its applicability to mass screening to select high-lysine corns from among germ plasm and breeding lines. It can be done with whole kernel or isolated endosperm extracts. For screening of individual seeds, the meal from a 5–10-mg endosperm slice is sufficient. Even an inexperienced person has no difficulty in distinguishing stained zein spots from normal and *opaque-2* corns (Fig. 1). The following is a protocol recommended for mass-screening:

1. Weigh 100 mg of meal into 13 × 10-mm Pyrex tubes.
2. Add 5 ml of 60% t-butanol, cap tubes, and suspend meal in the solvent by mixing in a vortex mixer for a few seconds.
3. Place tubes in a rack and shake on a rotary shaker at 150 rpm for 30 min.
4. Transfer to waterbath; heat at 70° C for 15 min.
5. Allow the particulate matter to settle (5 min).
6. Draw 5–10-μl aliquots with an automatic micropipet and spot at areas premarked (with a pencil) on paper. Also spot an aliquot each from normal and *opaque-2* corn extracts on the same paper. 200–250 samples may be spotted on the same sheet.
7. Stain for 15 min; destain for 5–10 min as described.
8. Inspect intensity of stained spots and mark those with staining intensity (potential high-lysine types) similar to that of the *opaque-2* corn.

For mass-screening purposes, the centrifugation step can be omitted because particulate matter settles to the bottom of the tube, leaving the clear supernatant at the top within 5 min. After the potential high-lysine types have been identified by the initial mass-screening, they can then be subjected to quantitative analysis (eg quantitative zein determination, Kjeldahl nitrogen determination, and amino acid analysis) to determine their exact status with respect to protein quality and quantity.

Limitations of the Method

The method in its present form cannot identify high-lysine/high protein genotypes because the amount of zein in such genotypes may be as much as that in normal corn. Similarly, the method cannot be used to predict lysine levels unless total protein and lysine contents of sufficiently large numbers of corn samples are determined and regression curves relating lysine content to zein content are established.

LITERATURE CITED

AMERICAN ASSOCIATION OF CEREAL CHEMISTS. 1962. Approved Methods of the AACC. Method 46-13, Approved October 1976. The Association: St. Paul, MN.

DALBY, A. 1974. Rapid method for determining the zein content of whole maize seeds or isolated endosperm. Cereal Chem. 51:586.

DUDLEY, J. W., ALEXANDER, D. E., and LAMBERT, R. J. 1975. Genetic improvement of modified protein maize. Page 120 in: High-Quality Protein Maize. Dowden, Hutchinson and Ross: Stroudsburg, PA.

ESEN, A. 1978. A simple method for quantitative, semiquantitative and qualitative assay of protein. Anal. Biochem. 89:264.

FAZEKAS de ST. GROTH, S., WEBSTER, R. G., and DATYNER, A. 1963. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. Biochem. Biophys. Acta 71:377.

FROMBERG, H. K., CHRIST, W., and POLLMER, W. G. 1971. A rapid method based on butanol extraction for selecting high-lysine maize (Zea mays L.). Crop. Sci. 11:567.

HERNANDEZ, H., and BATES, L. S. 1969. A modified method for rapid tryptophan analysis of maize. Centro Internacional de Mejoramiento de Maiz Y Trigo Res. Bull. 13.

JONES, R. A., DALBY, A., and TSAI, C. Y. 1975. A note on a modified technique for the rapid determination of zein content in maize. Cereal Chem. 52:279.

LOOMIS, W. D. 1974. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Page 528 in: CLOVICK, S. P. and KAPLAN, N. D. (eds.). Methods in Enzymology, Vol. 31. Academic Press: New York.

MERTZ, E. T., MISRA, P. S., and JAMBUNATHAN, R. 1974. Rapid ninhydrin color test for screening high lysine mutants of maize, sorghum, barley, and other cereal grain. Cereal Chem. 51:304.

MERTZ, E., NELSON, O., and BATES, L. S. 1964. Mutant gene that changes composition and increases lysine content of maize endosperm. Science 154:279.

MISRA, P. S., JAMBUNATHAN, R., MERTZ, E. T., GLOVER, D. V., BARBASO, H. M., and McWHERTER, K. S. 1972. Endosperm proteins synthesis in maize mutants with increased lysine content. Science 176:1425.

MISRA, P. S., MERTZ, E. T., and GLOVER, D. V. 1975a. Characteristics of proteins in single and double endosperm mutants of maize. Page 291 in: High-Quality Protein Maize. Dowden, Hutchinson, and Ross. Stroudsburg, PA.

MISRA, P. S., MERTZ, E. T., and GLOVER, D. V. 1975b. Studies on corn proteins: VIII. Free amino acid content of opaque-2 double mutants. Cereal Chem. 52:844.

MOSSBERG, R. 1969. Evaluation of protein quality and quantity by dye-binding capacity: A tool in plant breeding. Page 151 in: New Approaches to Breeding for Improved Plant Protein. International Atomic Energy Agency: Vienna.

PAULIS, J. W., WALL, J. S., and KWOLEK, W. F. 1974. A rapid turbidimetric analysis for zein in corn and its correlation with lysine content. J. Agric. Food Chem. 22:313.

VASAL, S. K. 1975. Use of genetic modifiers to obtain normal-type kernels with the opaque-2 gene. Page 197 in: High-Quality Protein Maize. Dowden, Hutchinson and Ross: Stroudsburg, PA.

ZUBER, M. S., and HELM, J. L. 1975. Approaches to improving protein quality in maize without the use of specific mutants. Page 241 in: High-Quality Protein Maize. Dowden, Hutchinson and Ross: Stroudsburg, PA.

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