Studies on Corn Proteins. V. Reduced Color Response of Opaque-2 Corn Protein to the Biuret Reagent, and its Use for the Rapid Identification of Opaque-2 Corn[1]

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ABSTRACT

The biuret method of Johnson and Craney for the determination of protein in corn (maize) gave reduced color values when applied to *opaque*-2 corn, a mutant with low zein content. Further tests with normal and *opaque*-2 corn protein fractions showed that the specific intensity of the biuret reaction is nearly four times greater with zein than with glutelin. Because of the reversal in the zein:glutelin ratio in *opaque*-2 corn, the biuret absorbance per unit of Kjeldahl nitrogen is 50 to 80% of that in normal corn. This color reduction can be used for the rapid identification of *opaque*-2 corn in corn breeding programs.

In 1964, Mertz and co-workers (1) reported that the *opaque*-2 (o_2) gene changed the protein composition and increased the lysine content of corn endosperm. Using a copper fractionation method (2), they found that the zein concentration of the o_2 endosperm is lower, and the glutelin concentration higher, than in normal endosperm. A second maize mutant with moderately increased lysine concentration, *floury*-2 (fl_2), was identified and reported in 1965 (3). In 1971, McWhirter (4) identified a third maize mutant with increased lysine concentration, designated *opaque*-7 (o_7). Recently, Misra and co-workers (5) reported that the zein levels of o_2, o_7, and the double mutants of o_2 with the starch-modifying mutants *sugary*-1 (su_1), *shrunken*-1 (sh_1), *shrunken*-2 (sh_2), *shrunken*-4 (sh_4),

[1]Journal paper 4745, Purdue Agricultural Experiment Station. Supported by Agency for International Development under contract "Inheritance and Improvement of Protein Quality and Content in Maize." Reprint requests should be directed to E. T. Mertz.

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brittle-1 (bt_1), and *brittle*-2 (bt_2) are much lower than zein level in the isogenic normal control, whereas fl_2 is only slightly lower.

In testing the Johnson and Craney (6) rapid biuret method for total protein on our corn samples, we obtained lower biuret absorbance values for *opaque*-2 than for normal corn. In this paper we show that the reduced biuret color response from *opaque*-2 corn is related to the reversed levels of zein and glutelin, and that this color reduction can be used for the rapid identification of *opaque*-2 corn.

MATERIALS AND METHODS

Corn Samples

Initial tests of the Johnson and Craney biuret method were made on the normal hybrid SX52 (Pfister Associated Growers) containing 1.72% nitrogen, and *opaque*-2 hybrid 50001, a near-isogenic line derived from normal SX52, containing 1.59% nitrogen. These two samples were also used for the Landry-Moureaux protein fractionations. The Ohio 43 samples analyzed by the biuret method consisted of near-isogenic sublines of the three floury (o_2, o_7,and fl_2) and six starch-modifying mutants listed above, plus amylose extender (ae), dull (du), sugary-2 (su_2), waxy (wx), and double mutants of all these genes except o_7 with o_2. Their derivation from inbred Ohio 43 has been described previously (5). The Ohio 43 series, as well as the W22 and W22/o_7 samples,were analyzed in the form of ground, defatted endosperms. These were obtained by soaking the dry corn kernels in distilled water for 30 min., separating them with a scalpel into pericarp, embryo, and endosperm, air-drying endosperms overnight, coarse grinding of the dry endosperms, defatting with hexane, and then grinding to a fine powder in a ball mill.

Whole kernels of different corn varieties were also analyzed by the biuret method either in the ground, undefatted form, or ground and defatted. A normal single cross of Ohio 43 and B14 and its near-isogenic counterpart homozygous for fl_2, five open-pollinated varieties from Mexico, one commercial hybrid from Brazil, five hybrids from Indiana and Illinois, and one Illinois inbred were included in the studies.

Biuret Method

Samples of finely ground corn, corn endosperm, or lyophilized corn fractions containing 20 to 150 mg. of protein as determined by micro-Kjeldahl were suspended in 2 ml. isopropyl alcohol; 1 g. powdered cupric carbonate was added, then 50 ml. alkaline alcohol solution (5.61 g. KOH plus 600 ml. isopropyl alcohol, plus water to make 1 liter). The mixture was shaken vigorously for 15 min., then allowed to stand for 15 min. to develop the biuret color. The mixture was filtered on a Gooch crucible and the clear filtrate collected. The absorbance of the filtrate was determined at 550 nm., and corrected for the absorbance obtained with a blank containing all components but the corn sample.

Fractionation of Corn Samples

Samples of SX52 and 50001 whole kernels were rough-ground, defatted with hexane, then ground to a fine powder in a ball mill. Five grams of the powder was extracted successively with the following solvents, according to the Landry-Moureaux extraction sequence D (7): Fraction I - 0.5M sodium chloride,

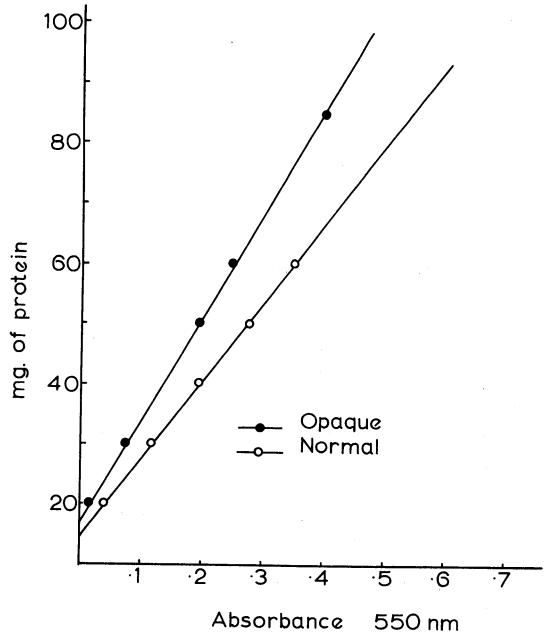

Fig. 1. Relationship of biuret absorbance values to Kjeldahl protein levels.

II - 70% isopropanol (v./v.), III - 70% isopropanol with 0.6% mercaptoethanol (2-ME) (v./v.), IV – borate buffer (μ 0.5, pH 10) with 0.6% 2-ME, and V- borate with 0.6% 2-ME and 0.5% sodium lauryl sulfate (w./v.). The alcohol in fraction II was evaporated on a steam bath, and the other fractions were dialyzed at 4°C. against three changes of distilled water at 12-hr. intervals. All fractions were then lyophilized. Nitrogen was determined on the lyophilized samples by the micro-Kjeldahl method, and the samples were then analyzed by the biuret method as described above.

RESULTS AND DISCUSSION

When samples of the two hybrids, SX52 and 50001, containing 21 to 84 mg. of protein, were analyzed by the Johnson-Craney biuret method, and the absorbance values at 550 nm. plotted against the mg. of protein used, the curves shown in Fig. 1 were obtained. The normal sample gave a straight line with a slope of 1.3×10^2, and the *opaque*-2 samples gave a straight line with a slope of 1.7×10^2.

When 800-mg. samples of the two hybrids were analyzed by the biuret method and the absorbance determined at wavelengths from 500 to 580 nm., the curves shown in Fig. 2 were obtained. This figure shows that the yield difference per unit of protein is not associated with a major shift in absorbance maximums. The *opaque*-2 corn absorbance value per unit of Kjeldahl nitrogen should have been 100% of that of the normal rather than 73%. We suspected that the difference in absorbance between these two samples might be the difference in zein content, and therefore fractionated the corn proteins using the Landry-Moureaux technique.

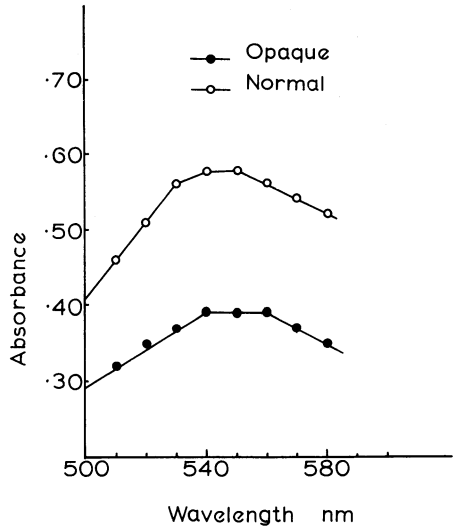

Fig. 2. Relationship of biuret absorbance values to wavelength of light used.

Ninety-five percent of the nitrogen was extracted in the normal corn and 100% in the *opaque*-2 corn. Table I shows the distribution of micro-Kjeldahl nitrogen among the five fractions. The level of fraction II (zein) is more than three times higher in the normal than the *opaque*-2 corn, and fraction V (glutelin) is 50% higher in the *opaque*-2 than in the normal corn.

Table II shows the absorbance values per mg. of nitrogen in fraction II (zein) and III (zein-like) are nearly four times the value in fraction V (glutelin), and two times the value in fractions I (albumin, globulins, free amino acids) and IV (glutelin). In *opaque*-2 (Table I), fractions I and V increased at the expense of fractions II and III, thus lowering the absorbance per mg. of nitrogen.

Data in Tables I and II show that the biuret absorbance values obtained from corn samples are related to the zein level. A standard curve is therefore needed for each zein level. Since the zein level is variable and lower than normal in corns containing the *opaque*-2 gene, the biuret method cannot be used for the rapid determination of protein. However, we have found that the reduced biuret color response of *opaque*-2 corn can be used for its rapid identification.

TABLE I. NITROGEN DISTRIBUTION IN WHOLE GRAIN CORN (MAIZE)

Fraction	Soluble Nitrogen (Percent of Total)	
	Normal	*Opaque*-2
I	16.6	39.0
II	38.6	11.9
III	10.1	6.2
IV	10.0	11.6
V	20.2	31.8
Total nitrogen extracted	95.5	100.5

Table III records the biuret absorbance values of a large number of endosperm samples expressed as a percent of the normal sample (Oh43+ or W22+). The biuret absorbance value of the isogenic *opaque*-2 was only 62% of that of the normal Oh43. In contrast, the *floury*-2 gene, which does not reduce zein levels appreciably (5), had no effect on lowering the biuret absorbance value. None of the nine starch-modifying mutant genes listed in Table III lowered the biuret absorbance value appreciably, even though some of them, such as the bt_2 gene, increased the lysine content to the level (3.3 g. per 100 g. protein) obtained with the *opaque*-2 gene. The Oh43 bt_2 endosperm is now being fractionated to determine why the biuret color is not reduced in this low-zein (5) mutant. The *opaque*-7 gene in the W22 background (5) reduces the biuret color to about 44% of the normal value (Table III), and thus appears to be the only mutant resembling o_2 in its response to the biuret reagent.

In contrast to the lack of response of fl_2 and the starch-modifying mutant genes alone, combination of any of these genes with the *opaque*-2 mutant gene reduces the biuret absorbance value (Table III). The *opaque*-2 gene is therefore effective in reducing the biuret color reaction not only when alone, but also when combined with other mutant genes.

The results of determining the biuret color value of both ground, undefatted and ground, defatted whole kernels obtained from different sources have been summarized in Table IV. Included in this table are inbred, hybrid, and open-pollinated varieties grown in the United States, Mexico, and Brazil. Using the Oh43 X B14 sample as the normal standard, all samples with 4.2% or more lysine gave biuret color values that were 50 to 76% of the normal control value.

These data show that when the *opaque*-2 gene is present in any of the backgrounds tested to date, the biuret color intensity per unit of protein is reduced to 50 to 80% of the normal corn from which it is derived. In contrast, the fl_2 gene and the nine starch-modifying genes studied do not have this color-reducing effect. In the one sample of *opaque*-7 corn studied, biuret color reduction resembled that observed with the *opaque*-2 gene.

TABLE II. RELATION OF BIURET ABSORBANCE TO NITROGEN IN THE LANDRY-MOUREAUX FRACTIONS

Sample	mg. N[a]	Absorbance[b]	Absorbance per mg. N
Fraction I			
Normal	4.99	0.08	0.016
Opaque	8.30	0.14	0.017
Fraction II			
Normal	6.20	0.25	0.043
Opaque	4.45	0.19	0.043
Fraction III			
Normal	4.65	0.21	0.045
Opaque	3.03	0.13	0.043
Fraction IV			
Normal	2.82	0.05	0.018
Opaque	3.70	0.09	0.024
Fraction V			
Normal	3.45	0.04	0.012
Opaque	3.95	0.05	0.013

[a]Micro-Kjeldahl method.

[b]Biuret color measured at 550 nm. (Bausch and Lomb Spectronic 20).

TABLE III. IDENTIFICATION OF *Opaque*-2 CORN IN THE OHIO 43 SERIES

Sample	Protein %	Lysine g. per 100 g. P	Sample mg.	Absorbance per mg. P	Percent of Normal
Oh43+[a]	11.4	1.7	800	0.0050	100
Oh43/o_2	9.4	3.3	800	0.0031	62
Oh43/fl_2	11.4	2.3	800	0.0053	106
Oh43/fl_2 o_2	11.2	2.8	800	0.0041	82
Oh43/ae	11.4	2.3	800	0.0048	96
Oh43/ae/o_2	10.9	3.9	800	0.0029	58
Oh43/du	10.7	2.3	800	0.0046	92
Oh43/du/o_2	10.0	3.7	800	0.0027	54
Oh43/sh_2	20.5	2.7	800	0.0056	112
Oh43/sh_2 o_2	18.7	5.3	750	0.0032	64
Oh43/sh_4	14.4	2.9	800	0.0059	118
Oh43/sh_4 o_2	9.5	4.1	800	0.0039	78
Oh43/su_2	11.5	1.9	800	0.0055	110
Oh43/su_2 o_2	9.7	4.0	800	0.0039	78
Oh43/wx	11.5	1.7	800	0.0055	110
Oh43/wx o_2	10.3	3.7	800	0.0034	68
Oh43/bt_1	11.9	2.6	800	0.0058	116
Oh43/bt_1 o_2	9.8	5.0	800	0.0024	48
Oh43/bt_2	13.4	3.3	492	0.0049	98
Oh43/bt_2 o_2	12.9	5.3	492	0.0032	64
Oh43/sh_1	13.1	2.2	800	0.0056	112
Oh43/sh_1 o_2	10.8	4.1	800	0.0033	66
W22+	9.4	2.3	800	0.0033	100
W22/o_7	7.7	3.8	800	0.0020	44

[a]All samples were ground, defatted endosperms. *Opaque*-2 are underlined in column 6.

TABLE IV. IDENTIFICATION OF *Opaque*-2 CORN IN DIFFERENT VARIETIES

Sample[a]	Origin	Protein %	Lysine g. per 100 g. P	Absorbance per mg. P	Percent of Normal
Oh43 X B14	Purdue	9.5	3.0	0.005	100
Oh43 X B14/fl_2	Purdue	9.3	3.3	0.0059	118
CM4854	Mexico	10.1	2.6	0.0046	92
CM4855	Mexico	10.1	5.0	0.0028	56
CM4856	Mexico	9.4	4.4	0.0031	62
CM4857	Mexico	9.7	4.7	0.0025	50
CM4858	Mexico	9.6	2.7	0.0047	94
Ag504	Brazil	11.3	4.4	0.0034	68
Hybrid	Indiana	13.0	2.8	0.0048	96
Hybrid	Indiana	11.1	4.2	0.0038	76
Hybrid	Illinois	8.7	4.5	0.0035	70
Hybrid	Illinois	9.3	3.3	0.0046	92
Hybrid	Illinois	9.8	4.2	0.0030	60
Inbred	Illinois	13.5	2.9	0.0057	114

[a]The first seven samples were ground, undefatted whole kernels. The remainder were ground, defatted whole kernels. *Opaque*-2 samples are underlined in column 6. In all cases, 800 mg. of finely ground corn was analyzed by the biuret method.

Since both the biuret reaction and the determination of protein are simple, rapid procedures requiring a minimum of equipment and technical skills, estimation of the biuret absorbance per unit of Kjeldahl nitrogen should be useful as a qualitative screening tool in the rapid identification of the *opaque*-2 character for high lysine

in corn breeding programs. This may prove especially important now that the *opaque*-2 endosperm is being altered by modifying genes to the extent that the high-lysine grain cannot be visibly differentiated from normal flint-type endosperm.

Acknowledgments

The authors wish to thank Miss Chi-Wan Chen and P. B. Shakya for technical assistance. The Mexican corn samples were kindly provided by the International Maize and Wheat Improvement Center, Mexico City, and the Brazilian sample by Sementes Agroceries, S.A., São Paulo.

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[Received May 8, 1972. Accepted September 25, 1972]