Biochemical and Agronomic Studies of Two Modified Hard-Endosperm *Opaque*-2 Maize (*Zea mays* L.) Populations[1]

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ABSTRACT

During the development of two hard-endosperm, *opaque*-2 (HEo_2) maize (*Zea mays* L.) populations, modifier genes were accumulated through several cycles of selection, to increase the proportions of vitreous and dense kernels (HEo_2 phenotypes). Lysine content of the modified materials was intermediate compared with normal and *opaque*-2 soft endosperm maize and was kept constant during agronomic improvement by a laboratory chemical screening procedure. Protein fractionation studies using a modified Landry-Moureaux procedure revealed that fraction I (albumins, globulins, and soluble nitrogen) content decreased, whereas fraction III (zeinlike) components increased parallel to cycles of selection. Characteristic of the modified HEo_2 structure was the high concentration of the zeinlike components, which increased in concentration as endosperm hardness of the population was improved through cycles of selection. No qualitative changes in any protein fraction components occurred during cycles of selection. A quantitative change in the proportion of two closely related zeinlike components was found during the later cycles of selection of one of the modified HEo_2 populations studied with polyacrylamide gel isoelectric focusing (PAGIF). Differences between zein and zeinlike polypeptides were established on the basis of their pI in PAGIF. Common components in both fractions suggested that they resulted from different subunit or molecular associations with other components.

Although the potential capabilities of the *opaque*-2 gene (Mertz et al 1964) for improving protein nutritional quality of maize (Maner 1975, Mertz et al 1965, Pradilla et al 1968) are well known, less than 1% of high-lysine maize types were grown in the United States in 1972. *Opaque*-2 cultivars have not been generally accepted by farmers in either the developed or lesser developed countries of the world because of the soft, chalky endosperm texture of the *opaque*-2 kernels, which generally results in lower kernel density, lower yield, and increased susceptibility to insects, pathogens, and mechanical damage.

Different approaches have been attempted by maize breeders to improve *opaque*-2 genotypes and include double mutant combinations (Glover et al 1975) and modifier genes of *opaque*-2 (Vasal 1975). The double mutant *sugary*-2/*opaque*-2 (*su₂o₂*) has outstanding protein quality, improved kernel density (Glover 1976; Misra et al 1972, 1975, 1976), and excellent nutritional value (Clark et al 1977, Graham et al 1980) but produces agronomically unacceptable small-sized grain and low yield (Glover 1976). *Brittle*-2 *opaque*-2 (*bt₂o₂*) has outstanding endosperm lysine content due to synergistic effects on zein synthesis, but it has a highly shrunken endosperm. In contrast, modifier genes described by Paez et al (1969) and Vasal (1975) lower the endosperm lysine content of *opaque*-2 accessions but noticeably improve the hardness of the endosperm and maintain seed quality. At the International Maize and Wheat Improvement Center in Mexico (CIMMYT), maize breeders have used recurrent selection for modifier genes to improve agronomic quality in different maize populations. Chemical screening for lysine content of harvested material in each cycle to develop hard endosperm *opaque*-2 (HEo_2) maize populations with improved lysine content has also been employed.

Biochemical studies of HEo_2 maize materials are few and contain several contradictory findings. Protein fractionation data for modified o_2 materials were obtained by using a diversity of fractionation schemes (Gentinetta et al 1975, Gupta et al 1979, Ribeiral 1973, Robutti et al 1974). Using the Landry-Moureaux (Landry and Moureaux 1970) fractionation scheme, Gentinetta et al (1975) demonstrated that the HEo_2 protein fraction mean values appeared to be intermediate between normal and *opaque*-2 materials. The HEo_2 strains were higher on the average in fraction III (zeinlike) percentage than either normal or *opaque*-2 lines.

In no studies were the maize genetic materials grown under uniform conditions and analyzed sequentially. The availability of seed from each cycle of selection from two of the most promising HEo_2 populations developed at CIMMYT stimulated us to investigate the agronomic and biochemical changes occurring as a result of modifier gene action over time and with respect to the endosperm protein components.

MATERIALS AND METHODS

Two hard-endosperm *opaque*-2 (HEo_2) populations of maize (*Zea mays* L.), Temperate × Tropical HEo_2 and CIMMYT HEo_2, were used. Seed from different cycles of selection from these accessions was increased at Tlaltizapán, México, under identical environmental conditions in the 1978B (May to October) cycle; the seed was harvested, bulked, and a subsample was used for this investigation.

Each maize population was physically characterized by its proportion of different kernel types according to phenotype and density (compared with sucrose solutions of different specific gravity). Four classes were differentiated on the basis of the proportions of hard and soft endosperm of each kernel: class A (>¾ soft); class B (½:½ soft–hard), class C (¼:¾ soft–hard) and class D (>¾ hard). Endosperm samples were prepared, finely ground and defatted (Hernández and Bates 1969), and analyzed for total nitrogen (AOAC 1965) and lysine (Moore and Stein 1951). Protein fractionation of duplicate 2-g endosperm samples from each cycle of selection and population was performed by following a slightly modified sequence D of the Landry-Moureaux (L-M) procedure (Landry and Moureaux 1970). Seven different fractions were obtained by refractionating L-M fraction I (0.3M NaCl solution) into albumins, globulins, and soluble nitrogen by means of a continuous diafiltration system (Blatt 1971) that included a model 52 Amicon cell with a UM-2 membrane filter and a stainless steel reservoir containing 0.003M NaCl solution.

To evaluate any change in the protein composition of each fraction during cycles of selection, L-M protein classes (low salt and SDS concentration) were characterized by isoelectrofocusing in polyacrylamide gel rods (Wrigley 1968). Isoelectrofocusing within a 3–10 pH gradient was used to characterize each of the protein classes: separated albumins and globulins from fraction I; true zeins; zeinlike; glutelinlike; and glutelins. Protein solutions from all the cycles of selection of each particular fraction were analyzed under identical conditions. Isoelectrofocusing was optimal with Biolytes™ for 15 hr at 5°C and a constant initial current flow/tube of 2 mA with no further adjustment. Urea (6M) was incorporated in the polyacrylamide gel to facilitate protein solubilization and migration (not necessary for albumin fraction).

Separated protein components were visualized with perchloric acid-Coomassie blue G reagent (Reisner et al 1975) for 30 min. Additionally, a minimum of 6 hr of fixation and staining was allowed for proper detection of the components present in lower concentration. Quantitative information was obtained by scanning the stained gel patterns with the use of a densitometer set at 640 nm and a recorder integrator system.

RESULTS AND DISCUSSION

Agronomic Changes

As previously reported for other modified o_2 materials (Paez et al 1969, Vasal 1975) these HEo_2 maize populations exhibited various endosperm phenotypes. Changes in the phenotypic composition during cycles of selection of both populations were similar. Selection progress for hard endosperm types via a recurrent selection scheme is represented in Fig. 1. The content of the desirable kernels (C + D) increased parallel to a decrease of soft type kernels in response to cycles of selection. Frequency distribution of Temperate × Tropical HEo_2 and CIMMYT HEo_2 kernels with different density through cycles of selection (Fig. 2b) demonstrated the effectiveness of the breeding scheme for improving kernel density of the populations.
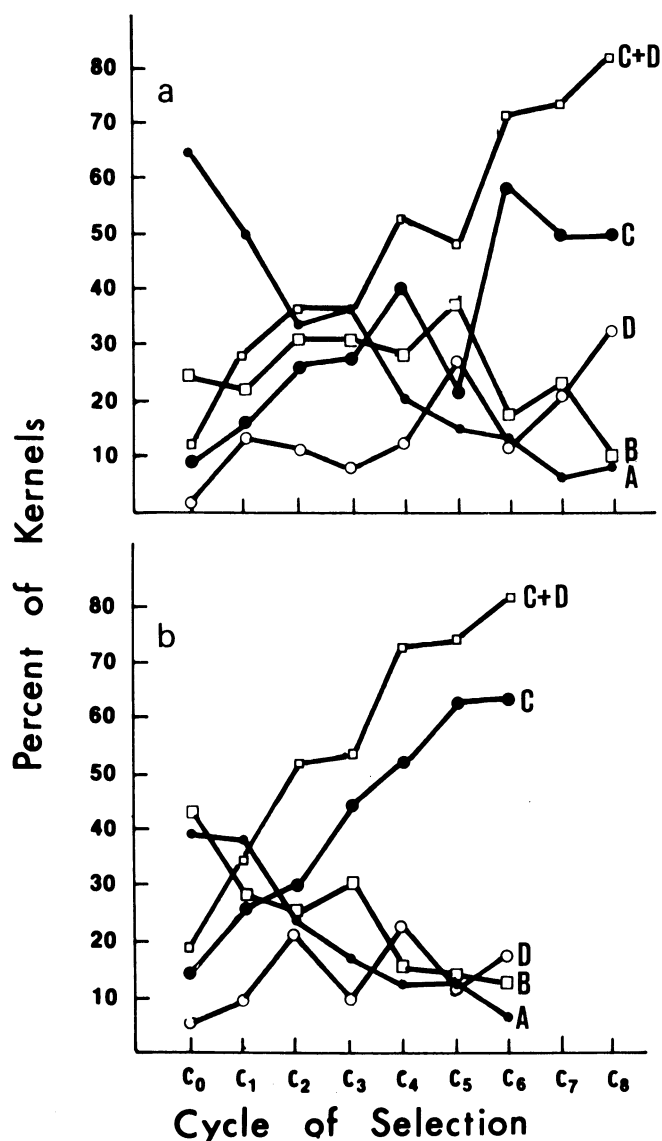
Biochemical Changes

Endosperm protein percentages ranging from 7.4 to 8.6 showed no apparent changes through cycles of selection (Table I). Lysine as a percent of protein (2.5–2.9) was intermediate to the normal (1.8) and opaque-2 soft material (3.6). No evident lysine concentration changes occurred through cycles of selection in either genotype (Table I).

Protein fraction distribution data are reported as percentage of total soluble nitrogen (Table II). Total nitrogen recovery ranged from 91 to 98% for the modified materials and nearly 98% in the normal and soft opaque-2 control samples. Nitrogen recovery was generally in the expected range (Gentinetta et al 1975, Ribeiral 1973) for HEo_2 materials. Protein fractions shifted as modifier genes accumulated in the populations. Fraction I content

Fig. 1. Changes in the proportion of different kernel classes through cycles of selection for two modified hard endosperm opaque-2 populations. a, Temperate × Tropical HEo_2; b, CIMMYT HEo_2. A, >¾ soft; B, ½:½ soft–hard; C, ¼:¾ soft–hard, and D, >¾ hard.
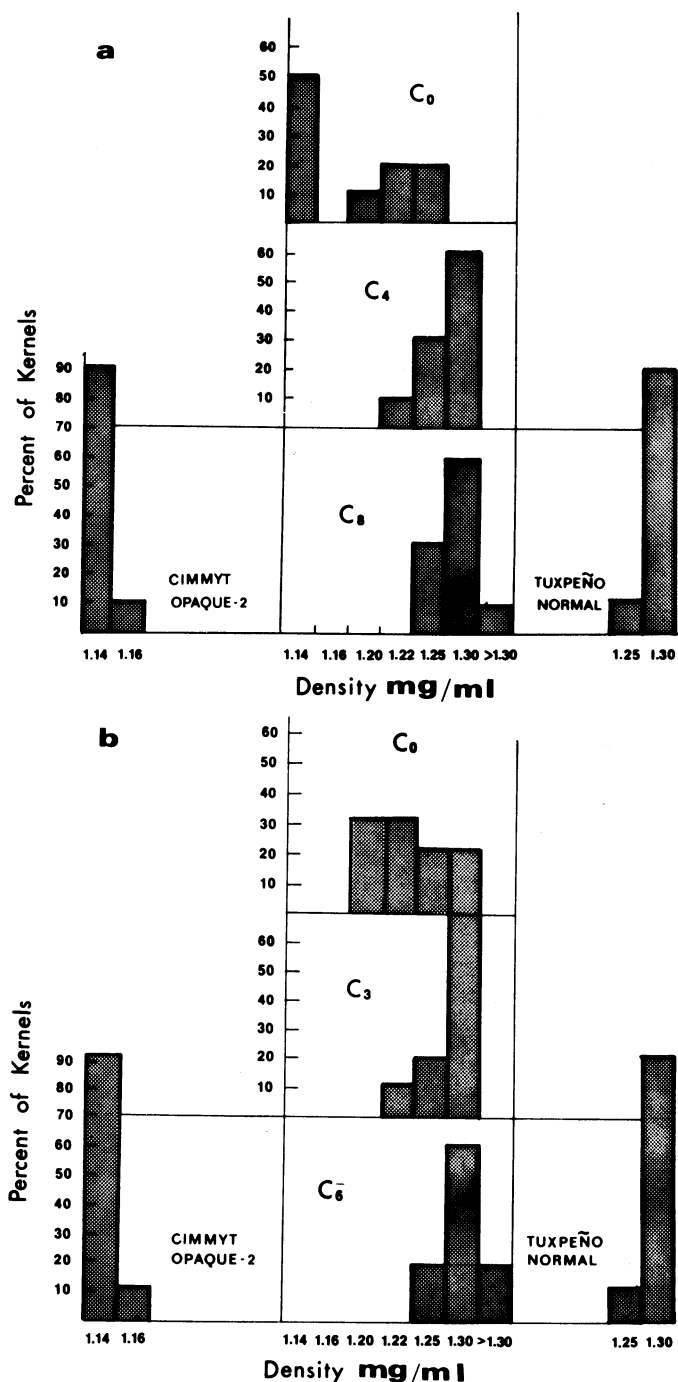

Fig. 2. Frequency distribution of modified hard endosperm opaque-2 kernels with different densities through cycles of selection compared to soft CIMMYT o_2 and Tuxpeño normal. a, Temperate × Tropical HEo_2; and b, CIMMYT HEo_2.

decreased, and fraction III content increased with cycles of selection in both populations. In contrast, fraction II (zein) changed irregularly, and fraction IV (glutelinlike) and fraction V (glutelin) content exhibited only minor changes and followed no particular trend. The decrease of fraction I (albumin-globulin) content correlates with the expected change in the transformation in maize of a soft *opaque*-2 to a hard endosperm type. Whereas *opaque*-2 soft cultivars are characterized by high amounts of fraction I, and hard normal cultivars have a very low content, HE*o2* samples have intermediate values. The characteristically high zeinlike (fraction III) content found for HE*o2* materials here and elsewhere (Gentinetta et al 1975) and the increase of this protein fraction, concomitantly with endosperm hardness of the populations studied, support a role for both components in the improved kernel texture.

Great heterogeneity was observed in all protein fractions analyzed by isoelectrofocusing as illustrated by a composite diagram (Fig. 3) of both HE*o2* population components from their last cycle of selection. The number of protein components detected ranged from 15 for the zein fraction to around 25 for the true glutelin (fraction V). The isoelectric point (pI) values for the different components ranged from 4.8 to 8.7. Each protein fraction exhibited a different distribution of components according to its pI and relative concentration, eg, the zein and zeinlike had 60–78% of the total components with a 6.5–8.2 pI range, whereas the albumin pattern had three acidic components that comprised about 50% of the total protein detected. The globulin pattern averaged 80% of the components with an acidic or neutral pI.

Qualitative differences between both maize populations were confined to the globulin and zeinlike patterns, particularly in the minor components. The similarity of the zein pattern for both populations suggests that specific genotype isoelectrofocusing

pattern differences reported previously for the zein fraction (Gentinetta et al 1975, Motto and Salamini 1979, Nucca et al 1978) result from different zeinlike components present in low concentration.

Quantitative differences through cycles of selection were found only in the zeinlike fraction for Temperate × Tropical HE*o2*. During the last three cycles of selection there was an increasing trend for one acid component (pI = 6.3) with a simultaneous decrease in a second component (pI = 6.1). This was visually detected in the stained pattern and data verified with densitometric analyses. The concentration changes of these two components seemed to be greatest at the initial and final stages of cyclic selection (Fig. 4). Each component was later resolved into two proteins (one major and one minor), respectively, with very similar pI in the 5–9 ampholyte range. Globulin solutions were achieved by using 10 M urea as a solvent for these proteins. Irregular globulin synthesis was observed through cycles of selection for both populations. In Temperate × Tropical HE*o2*, with the exception of the last cycle of selection (C8), the most acidic globulins (class A:pI 4.6–5.8) tended to increase, whereas most alkalines (class C:pI > 7.1), after reaching an early peak (C2), tended to decrease (Fig. 5). The significance of these data are questioned because of the very

TABLE I
Protein Concentration and Lysine in Protein Content of Temperate × Tropical HE*o2* and CIMMYT HE*o2* Endosperm Samples Through Cycles of Selection

Cycle of Selection	Temperate × Tropical HE*o2*		CIMMYT HE*o2*	
	Protein (%)[a]	Lysine (g/100 g protein)	Protein (%)[a]	Lysine (g/100 g protein)
C0	8.5	2.7	8.0	2.5
C1	8.2	2.8	8.3	2.7
C2	8.5	2.6	7.8	2.6
C3	8.2	2.7	8.6	2.6
C4	8.1	2.6	8.1	2.7
C5	8.0	2.6	8.1	2.5
C6	7.9	2.5	8.2	2.6
C7	8.2	2.6	...	...
C8	7.4	2.9	...	...

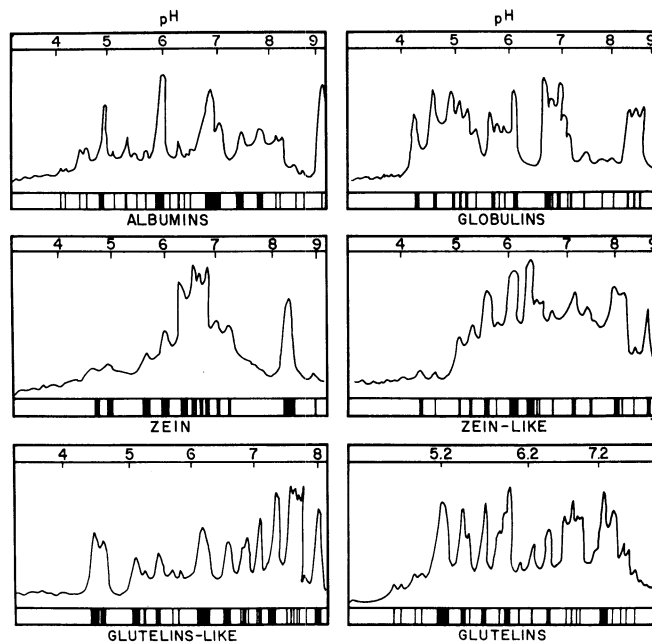[a] Percent nitrogen × 6.25; all values are means of two replications.

Fig. 3. Composite scanning densitometric pattern of the endosperm protein fractions separated by electrofocusing for both Temperate × Tropical HE*o2* and CIMMYT HE*o2* from their last cycle of selection.
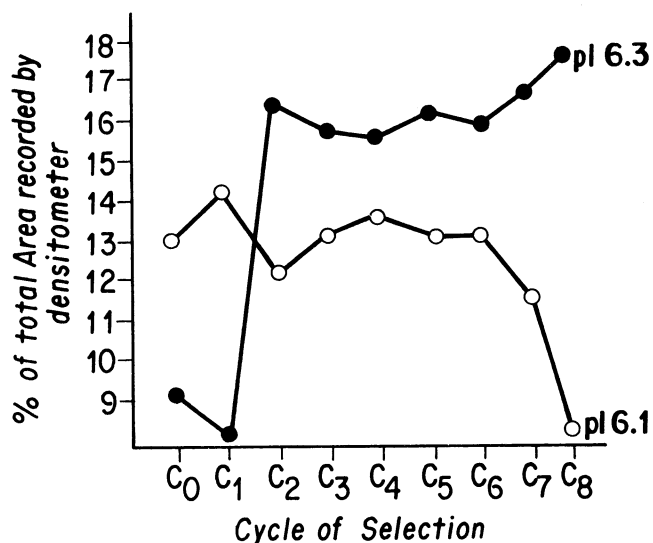
TABLE II
Changes in Endosperm Protein Fraction Distribution of Two Different Hard Endosperm *Opaque*-2 Populations Through Cycles of Selection

Cycle	Temperate × Tropical HE*o2*[a]					CIMMYT HE*o2*[a]				
	Fraction[b]					Fraction[b]				
	I Albumins Globulins	II Zein	III Zeinlike	IV Glutelinlike	V Glutelin	I Albumins Globulins	II Zein	III Zeinlike	IV Glutelinlike	V Glutelin
C0	15.4	18.1	20.0	16.3	30.2	16.8	17.6	16.2	19.7	29.6
C1	16.1	16.9	20.8	17.2	29.0	16.7	15.8	19.5	18.7	29.4
C2	15.8	18.5	21.6	17.5	26.5	16.9	15.2	15.4	20.3	32.1
C3	14.6	18.0	21.4	17.4	28.5	15.9	15.9	19.1	18.7	30.3
C4	14.6	16.7	21.7	19.2	27.8	14.9	13.8	21.8	18.9	30.6
C5	13.1	15.7	24.9	18.6	27.6	15.1	15.0	22.9	19.1	28.0
C6	13.1	16.2	24.0	19.9	26.8	13.9	16.1	23.5	17.5	28.9
C7	13.0	16.9	23.9	16.8	30.0	...	...	...	...	...
C8	12.5	13.1	24.1	19.0	31.4	...	...	...	...	...

[a] Mean values of two replications refer to percentage of total soluble nitrogen.
[b] Modified Landry-Moureaux (1970) fractionation procedure.

Fig. 4. Changes in the content of two main zeinlike components pI 6.1 = o——o; pI 6.3 = •——• of Temperate × Tropical HE*o2* endosperm samples through cycles of selection.
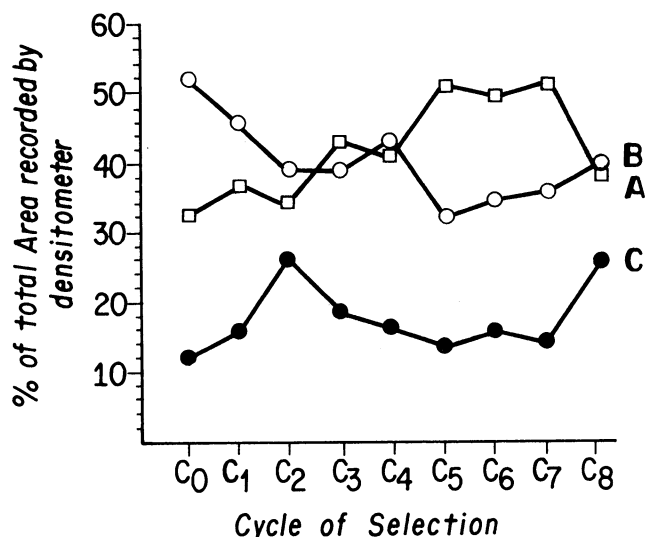
Fig. 5. Changes in the proportion of globulins with different isoelectric points, class A (□——□), B (o——o), and C (•——•), of Temperate × Tropical HE*o2* through cycles of selection.

irregular response observed for the different globulin classes, in particular for class B globulins having a pI of 5.85 to 7.1 (Fig. 5).

Zeinlike protein patterns differed from the zein patterns by the presence of certain bands. This is in agreement with previous findings using polyacrylamide gel electrophoresis (Sodek and Wilson 1971) and isoelectrofocusing (Righetti et al 1977, Soave et al 1975). Five components in the zein fraction were not detected in the zeinlike fraction (Fig. 3). Similarly, six components were typical of the zeinlike fraction. This was confirmed when all components in either zein or zeinlike protein solutions were adequately detected by scanning densitometry in a gel containing a 1:1 mixture of both solutions. Even though some zein and zeinlike polypeptides are identical (on the basis of their pI), their presence in both fractions can be explained only as a consequence of a different molecular association with typical zeinlike components or possibly with other types of proteins (probably glutelin) within the endosperm structure of the kernel.

CONCLUSIONS

Two maize populations containing the *opaque*-2 gene were successfully converted by CIMMYT maize staff to hard endosperm types with intermediate protein quality compared to normal and soft *opaque*-2 types and with improved agronomic characteristics after recurrent selection in the field and chemical laboratory screening. Physical characteristics such as the proportion of hard endosperm kernels with increased density demonstrated the effectiveness of this procedure. Protein content of the endosperm showed no change or only a slight decrease as modifier genes were accumulated through cycles of selection.

Endosperm lysine as percent of protein of composite samples from both populations studied ranged from 2.5 to 2.9, values intermediate between those of *opaque*-2 and normal cultivars. Endosperm lysine concentration of both populations remained essentially stable through cycles of selection due to selection based on chemical composition, as well as for hard endosperm.

Protein fractionation studies of Temperate × Tropical HE*o2* and CIMMYT HE*o2* through all the cycles of selection demonstrated that protein shifts occurred as modifier genes were accumulated through cycles of selection. Fraction I content decreased and fraction III content increased with cycles of selection. The decrease in the albumin, globulin, nitrogen-soluble components (fraction I) was explained as a consequence of the protein concentration change expected when modifying a soft *opaque*-2 to a hard normal phenotype kernel. The increased concentration of fraction III (zeinlike) along with the specific, higher content of these proteins in modified materials indicate that the protein shift is controlled by modifier genes associated with the improved hard kernel texture of the populations.

Using isoelectrofocusing, we identified and characterized all protein components in modified HE*o2* endosperm samples on the basis of their isoelectric points. We observed no qualitative changes in the albumin, globulin, zein, zeinlike, glutelinlike, or glutelin protein patterns with cycles of selection in either population. Quantitatively, the only change observed was in the proportion of two zeinlike components during the later cycles of selection in Temperate × Tropical HE*o2*.

Differences between fraction II (zein) and fraction III (zeinlike) polypeptides were specifically established on the basis of pI. Common components in both fractions suggested their existence as part of different subunit associations or in different molecular association with other components.

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