Variability of α-Amylase Synthesis in Germinating Maize

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ABSTRACT

α-Amylases from germinating seeds of five maize genotypes were isolated by affinity chromatography and chromatofocusing for comparison with enzymes from cultivar B73, which contains four major groups of enzymes separable by these techniques: B-I and B-II have elution pH greater than 5.0; B-III has elution pH of 4.5–4.8; and B-IV has elution pH of 4.0–4.1. These four groups exhibit three specific patterns of hydrolysis on soluble starch, differing primarily in the proportion of degree of polymerization (DP) 2 and DP6 oligosaccharides produced. Enzyme groups found in other cultivars were the same as those found in B73, but the amount of synthesis of the different groups varied substantially. Enzymes from each cultivar were evaluated for hydrolysis of soluble starch. Four of the genotypes contained substantial amounts of enzyme fractions with an elution pH in the range of 4.3–4.5 but with an action pattern different from that of B-III; these fractions appeared to be unable to hydrolyze oligosaccharides smaller than DP8.

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We recently reported separation of the multiple forms of α-amylases found in germinating maize cultivar B73, using affinity chromatography and chromatofocusing (Warner et al 1991). These enzymes separate into two groups according to their affinity for cycloheptaamylose (CHA)-sepharose. Only the enzymes that bind to CHA-sepharose, constituting 45–55% of total activity, were found to be effective at attacking starch granules. Four major fractions of the affinity-bound amylases were identified by chromatofocusing, isoelectric focusing (IEF), and reaction product analysis on soluble starch substrate. These groups were designated B-I, B-II, B-III, and B-IV; "B" signifies that they are bound by affinity chromatography, and "I–IV" designates their relative order of chromatofocusing elution. B-I and B-II groups both have chromatofocusing elution pHs greater than 5.0. IEF revealed that these groups, which comprise the greatest portion of total activity and total protein, are not completely separated by chromatofocusing. Fractions were later obtained that are enriched in each of the major enzymes of each group (Warner and Knutson 1991), but complete separation has not yet been achieved. Group B-III α-amylase, which is relatively pure, elutes in the pH range of 4.7–4.8 and contains a single active IEF band. Group B-IV, which is also relatively pure, elutes at pH 4.0–4.1 and shows an active IEF doublet band.

Reaction product analysis shows that these enzyme groups exhibit three characteristic action patterns. Groups B-I and B-II exhibit the classic pattern for cereal α-amylases, i.e., they primarily produce oligosaccharides with degree of polymerization (DP) of 2 and 6 in nearly equal amounts. Group B-III α-amylase exhibits an unusual action pattern for cereal α-amylases—at the reaction limit, the product mixture is primarily DP2 with very little DP6. Group B-IV has an action pattern similar to that of B-I and B-II, except that the proportion of DP6 is 50% greater than that of DP2. This latter enzyme group is the most active on granular starch.

The comparison of our chromatofocusing results with those of MacGregor et al (1988) suggests substantial qualitative and quantitative differences in α-amylase content among maize genotypes. To investigate this variation further, we isolated the affinity-bound α-amylases from other maize cultivars. Preliminary examination of α-amylases from numerous cultivars by IEF showed essentially the same bands present in all cultivars but indicated substantial quantitative variation. On the basis of this preliminary study, five cultivars were chosen for more extensive evaluation. Samples selected for further evaluation were cultivars that grow in arid climates and cultivars developed for cold tolerance, on the premise that genotypes adapted to extreme conditions would be most likely to exhibit variations in enzyme synthesis, if such variations exist. Due to the nature of their habitat, these cultivars must germinate and begin to grow quickly when conditions of moisture and temperature are suitable. As a result of these constraints, these cultivars could be expected to initiate enzyme synthesis rapidly, to produce greater amounts of enzyme, and possibly to produce different enzyme forms as a means of facilitating starch hydrolysis and enhancing the survival of the emerging seedling.

MATERIALS AND METHODS

Maize Samples

Cultivar B73 was obtained from Illinois Foundation Seeds, Inc. Three cold-tolerant cultivars (BSCT, MESS, and NAQT) and a cultivar grown in the northwest desert of Mexico (Zapalote Chico) were provided by Linda Pollak, Midwest Area Cereal and Soybean Improvement Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Agronomy Department, Iowa State University, Ames, IA. Alamo blue maize was provided by Jim Wolf of the Alamo Navajo School Board Vocational Education Program, Magdalena, NM.

Seed Germination and Enzyme Extraction

Samples of 100 kernels of each maize cultivar germinated in the dark for seven days at 25°C, and crude enzyme extracts were prepared using pH 5.0 acetate buffer as described by Warner et al (1991). The final volume of the extract was 10 ml; the solution thus contained enzyme from 10 kernels in each milliliter.

Affinity Chromatography

All liquid column chromatography was performed using a Pharmacia fast protein liquid chromatography system. Affinity chromatography of the acetone-precipitated proteins was performed on cycloheptaamylose (CHA)-epoxy sepharose 6B at pH 5.0 according to the method of Silvanovich and Hill (1976), using enzyme extract from 100 kernels (i.e., 10 ml of sample).

Chromatofocusing

The affinity-bound fractions were further purified by chromatofocusing on a Pharmacia Mono P column as described by Warner et al (1991) over a pH range of 6.5 to 3.5. One-milliliter fractions were collected. Elution pH, approximately equivalent to the isoelectric point (pI), was monitored with a flow-through pH electrode or by measuring the pH of individual fractions with a Beckman Expandomatic pH meter (Beckman Instruments, Fullerton, CA) equipped with a combination electrode.

Enzyme Activity

Amylase activity was measured using the dinitrosalicylic acid assay (Bernfeld 1951) with maltose as a standard. One unit of activity was equivalent to 1 μmole of maltose released per minute.

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Analysis of Reaction Products

Individual fractions from chromatofocusing were incubated with soluble starch in 20 m*M* acetate buffer at pH 4.5; 200 μl from each 1-ml enzyme fraction was added to 10 ml of 4% (w/v) starch solution. At intervals between 0.5 and 144 hr, an aliquot was removed from the reaction mixture, mixed with two volumes of absolute ethanol, centrifuged to separate the residue, and filtered through a 0.2-μm filter. Composition of reaction mixtures was determined by high-performance liquid chromatography, using a Spectra-Physics (San Jose, CA) SP8100 high-performance liquid chromatograph equipped with a Dionex (Sunnyvale, CA) PAD-2 pulsed amperometric detector and a Dionex Carbopak PA1 carbohydrate column. Oligosaccharides were separated by gradient elution at 40°C. Mobile phases were 100 m*M* NaOH (solution A) and 100 m*M* NaOH plus 500 m*M* sodium acetate (solution B); the gradient proceeded from 98% A to 70% A in 5 min, then to 50% A at 15 min, and was maintained at that point for an additional 5 min. Flow rate was 1 ml/min. Maltooligosaccharides DP1 to DP7 from Sigma Chemical Company (St. Louis, MO) were used as standards.

RESULTS

Comparison of Enzyme Activity Levels

Activity varied significantly among the cultivars, both on a per-kernel basis and on per-unit weight. Endosperm weights (milligrams per kernel) and crude amylase activity (units per gram of endosperm, duplicate analysis) after seven days of germination were as follows: B73 (227, 24.8); Alamo (190, 57.8); BSCT (204, 60.4); MESS (132, 52.4); NAQT (124, 24.1); and Zapalote Chico (160, 27.1). Total crude extract from a 100-kernel sample was used for affinity chromatography and chromatofocusing for each cultivar.

Characteristics of Control Sample B73

Chromatofocusing elution of B73 affinity-bound α-amylases is shown in Figure 1. The major peaks are labeled B-I through B-IV in order of their elution from the Mono P column. Final reaction product mixtures, i.e., mixtures found at the limit of hydrolysis, for these enzyme fractions acting on soluble starch substrate are shown in Figure 2. Patterns for B-I (Fig. 2A) and B-II (Fig. 2B) were indistinguishable from one another, and the pattern for B-IV (Fig. 2D) was quite similar, except that the ratio of DP2 to DP6 is lower. B-III (Fig. 2C) samples displayed the most unusual pattern; the final product mixture was predominantly DP2, with very low amounts of DP1 and DP6. These reaction patterns were found to be consistent among all the cultivars used in this study, and no significant variations in final reaction product mixtures were observed with varying enzyme concentration, provided that the activity level was high enough to give measurable quantities of reaction products in 6 hr or less.

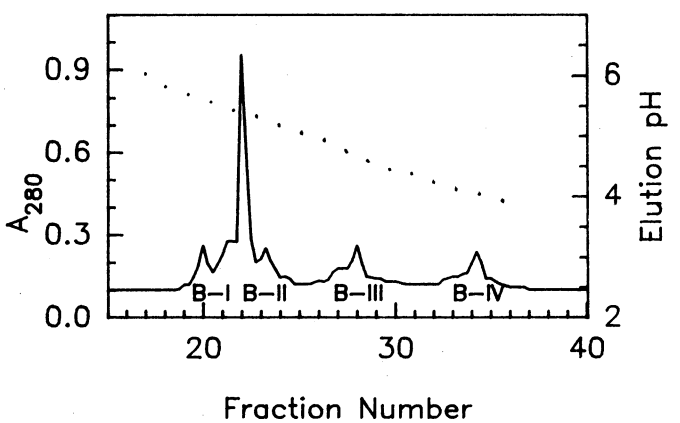

Fig. 1. Chromatofocusing elution profile of affinity-bound α-amylases from maize cultivar B73, showing fractions B-I through B-IV. Solid line is absorbance at 280 nm; dotted line is elution pH.

Characteristics of Other Cultivars

Chromatofocusing elution profiles of the other five maize cultivars are displayed in Figure 3. Profiles had general similarities, but each cultivar had significant variations that resulted in a characteristic profile for that cultivar. Action patterns of individual peaks were determined on soluble starch substrate, and those peaks with elution pH and action patterns corresponding to the B-I through B-IV patterns of cv. B73 were labeled accordingly. In addition to peaks that could be labeled with respect to B73, several cultivars contained undefined peaks in the pH 4–5 range that had not been observed in cv. B73. Major differences exhibited by individual cultivars are described below.

Alamo (Fig. 3A) produced the largest amount of activity, with a very high content of B-III. Additionally, this cultivar contained a substantial undefined peak eluting at pH 4.4. BSCT (Fig. 3B) displayed a series of small peaks in the B-III region at pH 4.8, 4.6, and 4.5, none of which could be positively identified as B-III

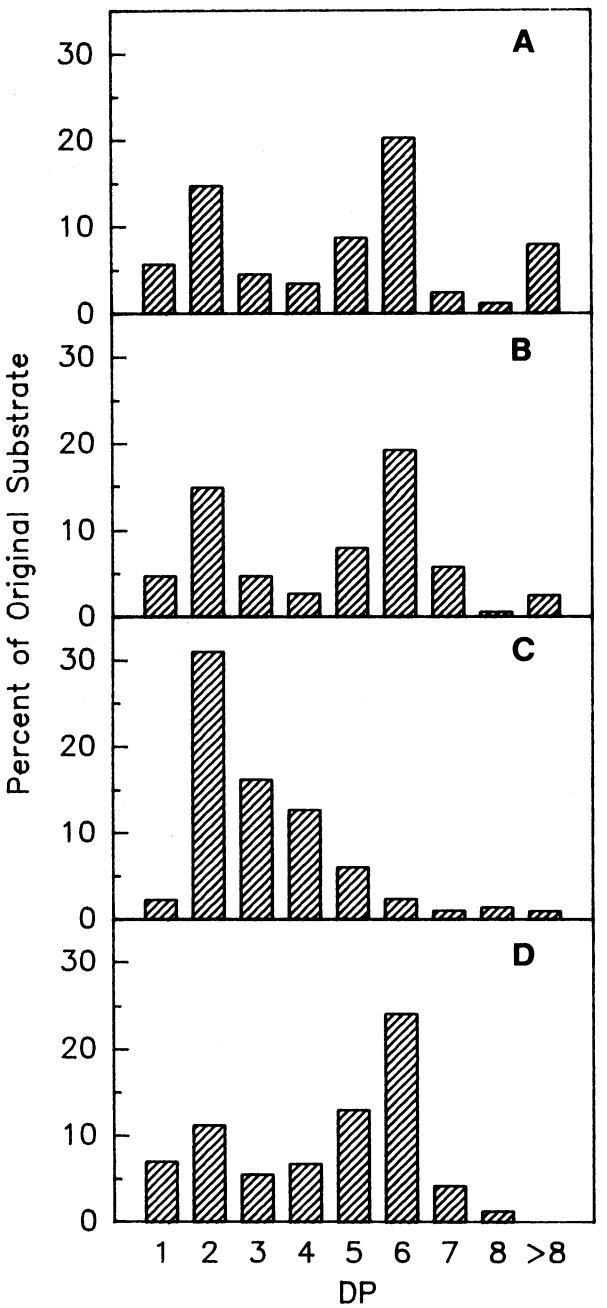

Fig. 2. Action patterns of affinity-bound maize α-amylases. **A,** Chromatofocusing fraction B-I; **B,** fraction B-II; **C,** fraction B-III; **D,** fraction B-IV. Y-axis represents weight percent of original substrate weight found in each oligomer. DP = degree of polymerization.

from the chromatogram alone; subsequent action pattern analysis determined that the B-III peak was that with an elution pH of 4.5. MESS (Fig. 3C) had a poorly resolved undefined peak at pH 4.4. NAQT (Fig. 3D) had a large area of poorly resolved peaks at pH 4.5, 4.4, and 4.3; none of these could be positively identified as B-III by action pattern analysis.

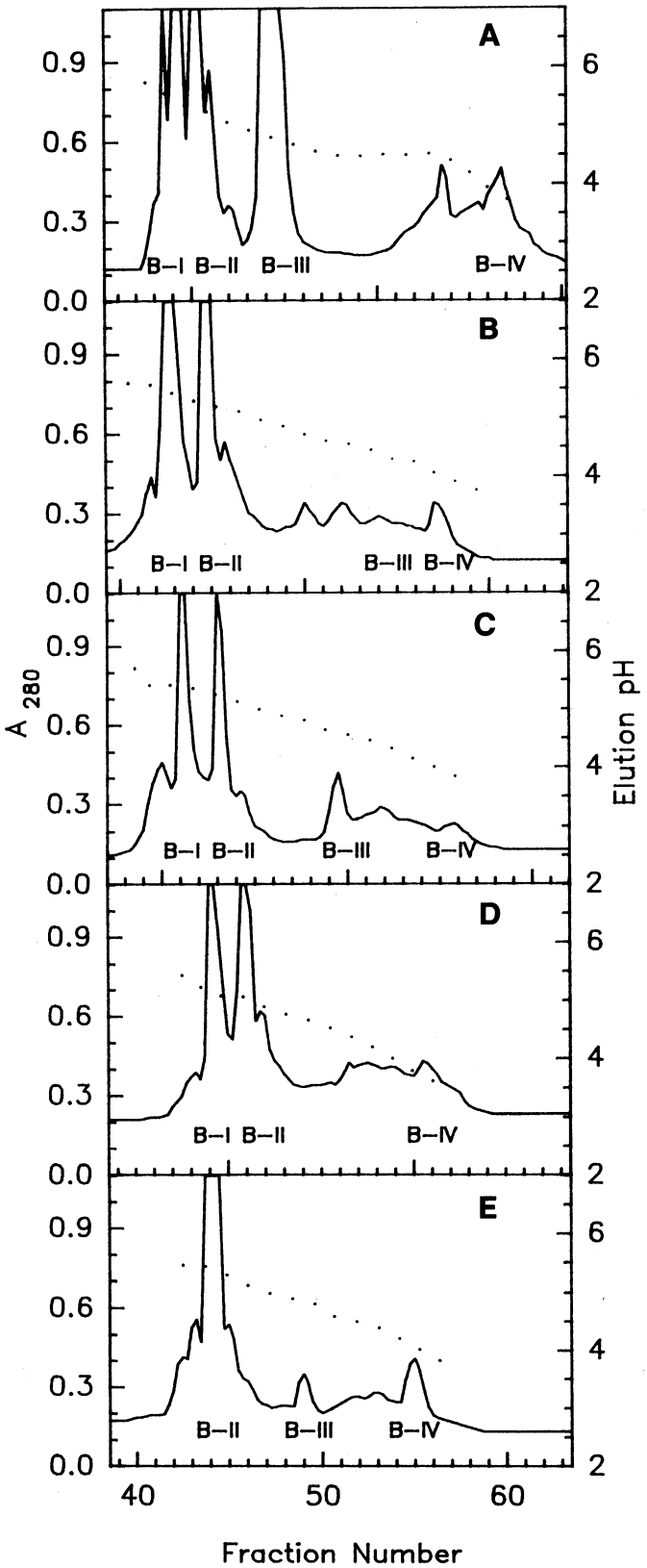

Fig. 3. Chromatofocusing elution profiles of affinity bound α-amylases from five maize cultivars: **A**, Alamo blue; **B**, BSCT; **C**, MESS; **D**, NAQT; **E**, Zapalote Chico. B-I, B-II, B-III, and B-IV are enzyme fractions.

Zapalote Chico (Fig. 3E) contained a single large peak in the B-II region at pH 5.4, with only a minor amount of B-I present in a shoulder at 5.5, poorly resolved peaks at 4.3–4.5, and a relatively large B-IV peak.

In all of the cultivars, those peaks resolving at intermediate pH (approximately 4.3–4.8) that did not display the B-III action pattern displayed the pattern shown in Figure 4. These "non-B-III" fractions were similar to B-III inasmuch as they produced very little glucose, but they appeared to be incapable of hydrolyzing oligosaccharides of DP 6 and 7. The non-B-III component was present in very small amounts in MESS, NAQT, and Zapalote Chico and had not been identified previously in cv. B73 (Warner et al 1991). Careful reevaluation of B73 fractions revealed a small proportion of non-B-III activity.

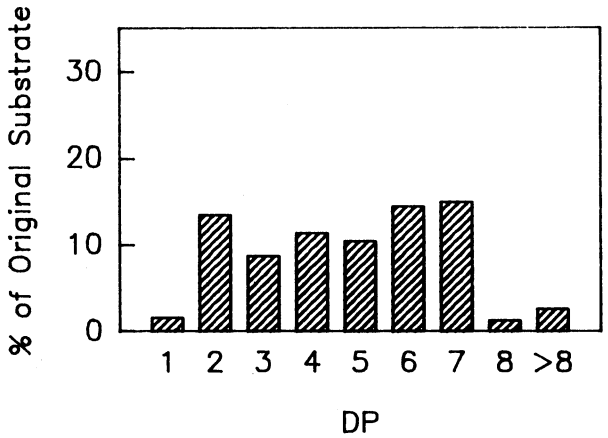

Fig. 4. Action pattern of intermediate pI "non-B-III" α-amylase. Y-axis represents weight percent of original substrate weight found in each oligomer. DP = degree of polymerization.

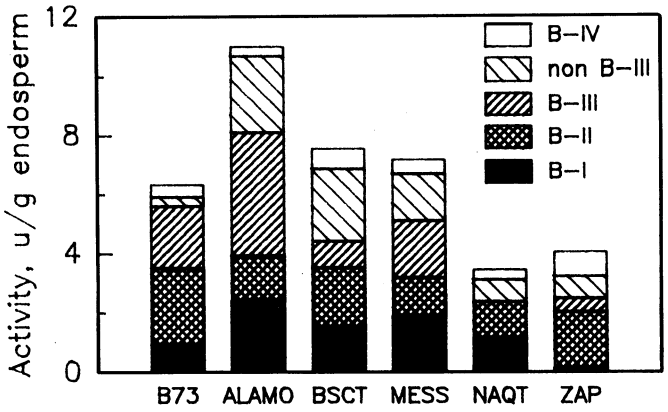

Fig. 5. Total activity and activity of individual fractions from six maize cultivars. B-I, B-II, B-III, and B-IV are enzyme fractions.

TABLE I
Elution pH of Affinity-Bound Chromatofocusing Fractions of Maize α-Amylases

	Fraction				
Cultivar	**B-I**	**B-II**	**B-III**	**non-B-III**	**B-IV**
B73	5.53	5.36	4.76	4.57	4.10
Alamo	5.57	5.32	4.73	4.48	3.82
BSCT	5.52	5.22	4.38	4.64	3.89
MESS	5.49	5.26	4.52	4.67 4.25	3.90
NAQT	5.22	5.13	...	4.46	3.80
Zapalote Chico	5.46	5.28	4.82	4.48	3.97
Average	5.46	5.26	4.64	4.51	3.91
SD	0.13	0.08	0.18	0.14	0.11

Comparison of Total Activity of Maize Genotypes

Figure 5 shows a comparison of the amount of activity of each action type and total activity for the genotypes used in this study. Substantial variation in total activity and in activity of individual enzyme types was found.

Elution pH values for major fractions from each cultivar are listed in Table I, along with mean elution pH and standard deviation for each enzyme group. Analysis of variance and Duncan's multiple range test indicated that the means of elution pH for the individual groups differed significantly at the 0.01 level, except for B-III and non-B-III, which differed at the 0.05 level.

DISCUSSION

In previous studies, we have used two alternative isolation procedures for maize α-amylases. In the original procedure (Warner et al 1991), all soluble proteins extracted from endosperm were precipitated with acetone, which denatured and insolubilized major amounts of inactive proteins. Active proteins were redissolved and fractionated by affinity chromatography to isolate α-amylases, followed by chromatofocusing. Approximately half of the initial activity was recovered by redissolving the acetone precipitate. The second procedure (Warner and Knutson 1991) preserved a higher proportion of initial activity by using hydroxylapatite chromatography rather than acetone precipitation to remove inactive proteins. Active proteins then were fractionated by chromatofocusing, which permitted simultaneous recovery of α-amylases, β-amylases, and debranching enzymes. α-Amylases from chromatofocusing fractions then were isolated by affinity chromatography.

For this study, it was advantageous to use the simpler original isolation procedure, because our ultimate interest was in α-amylases that readily attack starch granules; those maize α-amylases that do not bind to CHA are ineffective for hydrolysis of granular starch (Warner et al 1991). To ascertain that this procedure did not adversely affect the recovery of the enzymes of interest, a sample of crude B73 endosperm extract was precipitated with acetone, and an identical sample was separated by hydroxylapatite chromatography. Both samples then were subjected to affinity chromatography followed by chromatofocusing. The samples gave identical chromatofocusing patterns and activity levels (data not shown), which demonstrated that any activity lost by acetone precipitation was not from affinity-bound α-amylases. Therefore, acetone precipitation was used routinely for isolation of enzymes in this study.

Affinity-bound enzymes are assumed to be totally α-amylase, on the basis of earlier findings, as follows. First, maltase and debranching enzyme activity is found in crude extracts but not in bound fractions from affinity chromatography (Warner et al 1991). Second, in eluates containing mixtures of carbohydrases with similar elution pH, only α-amylases bind to a CHA affinity column (Warner and Knutson 1991). And third, IEF patterns of affinity bound fractions are identical with either starch or β-limit dextrin as substrate in the detection gel, indicating the absence of β-amylase (Warner et al 1991). As further verification, we recently demonstrated that the action patterns of affinity-bound chromatofocusing fractions were unchanged when fractions were heated at 70° C for 15 min, precluding the presence of β-amylases contaminants (C. A. Knutson and M. J. Grove, *unpublished data*).

Total activity, proportion of activity bound by CHA, and activity level of individual enzyme types varied substantially among these genotypes. The proportion of crude activity recovered as affinity-bound α-amylase varied from 12 to 26% among the cultivars. Presumably, these differences were partly caused by differences in the degree of synergism between β-amylases and other affinity-unbound enzymes; recovery of activity of affinity-bound enzymes after chromatofocusing was nearly quantitative. B-II and B-IV enzymes were identified in all cultivars. Activity of B-IV, the fraction shown to attack granular starch most rapidly (Warner et al 1991), was consistently lower than that of the high pI enzymes and, in most cases, was lower than that of the intermediate pI enzymes as well. It appears that a relatively small proportion of this enzyme is required for granular attack during germination. Other α-amylases may act cooperatively with B-IV to attack granules that have been initially invaded by B-IV. Further study is needed on the cooperative or synergistic nature of these enzymes in granular starch hydrolysis.

The similarity of the action pattern for B-I and B-II enzyme groups reflects the fact that these components are incompletely resolved, as demonstrated by IEF banding patterns. However, because no difference in the B-II pattern was observed in the Zapalote Chico sample, in which the B-I component was negligible, it appears likely that little difference exists in the action of these two forms.

The similarity in elution pH between the B-III fractions and the non-B-III intermediate pI fractions, as well as the fact that neither group produces significant quantities of glucose, indicates a probability that these forms are closely related; the non-B-III form may be inhibited in some fashion, preventing it from attacking DP6 and DP7 oligosaccharides. Alternatively, on the basis of elution pH and action pattern, the non-B-III form may be similar to the B73 α-amylases that do not bind to CHA (Warner et al 1991). This form may be generally present, but its CHA binding inhibited to varying degrees in different genotypes. Further investigation of this form is needed, especially investigation regarding its ability to attack granular starch.

Quantitative variation in enzyme synthesis was substantial in this small sample. However, no evidence was found that cultivars capable of growth under adverse conditions synthesize any different enzymes, nor have higher rates of synthesis, than do genotypes developed for the traditional corn-growing regions.

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