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Allelic Studies of the *Amylose-Extender* Locus of *Zea mays* L.: Levels of the Starch Branching Enzymes

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INTRODUCTION

Starch branching enzymes (α-1,4-glucan:α-1,4-glucan-6-glycosyl transferase; EC 2.4.1.18) catalyze the formation of α-1,6 linkages in starch. The reaction proceeds by the hydrolysis of a 1,4 bond, followed by the subsequent reattachment of the released 1,4-glucan chain to the remaining or to another 1,4-glucan chain by a 1,6 bond. This creates branch points in the starch molecule as well as additional nonreducing ends where further synthesis of starch can occur. Multiple forms of branching enzyme have been identified and characterized in maize endosperm (Boyer and Preiss, 1978a; Hodges *et al.*, 1969), pea cotyledons (Matters and Boyer, 1981), and spinach leaf extracts (Hawker *et al.*, 1974) by DEAE-cellulose and aminoalkyl Sepharose chromatography, disc-gel electrophoresis, molecular weight, activity in various buffers, type of α-glucan product formed, and the ratio of two different assay procedures (units of phosphorylase *a* stimulation divided by units of decrease in absorbance of the amylose–iodine complex).

The *amylose-extender* (*ae*) mutant in maize was first observed by Vineyard and Bear (1952) and has been found to be associated with a characteristic increase in the amylose content of endosperm starch to approximately 60% (Deatherage *et al.*, 1954). Gene dosage studies by Ferguson *et al.*

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(1966) revealed a corresponding increase in the amylose content of endosperm starch. Boyer and Preiss (1978b) reported *ae* endosperm extracts to contain no detectable quantities of branching enzyme IIb, while the levels and properties of branching enzyme I and IIa were unaltered. Baba *et al.* (1982) recently confirmed these observations, while recent work by Hedman and Boyer (1982) demonstrated a near-linear relationship between increasing gene dosage of the dominant *Ae* allele and branching enzyme IIb activity. In contrast, the levels and properties of branching enzymes I and IIa, as well as the citrate-stimulated and primed starch synthases, were shown to be unaffected by *ae* gene dosage. These results have led to the hypothesis that the dominant *amylose-extender* allele is the structural gene for branching enzyme IIb (Hedman and Boyer, 1982).

The research presented here demonstrates that endosperm extracts from six independently derived *ae* mutants exhibit a deficiency in branching enzyme IIb activity. Allelic substitution at the *ae* locus was determined to have no effect on the levels and properties of branching enzymes I and IIa and the starch synthases. These results support the hypothesis that *ae* is the structural gene for branching enzymes IIb.

MATERIALS AND METHODS

The maize (*Zea mays* L.) dent inbred W64A (*Ae*) and the W64A backcross conversions of the *amylose-extender* alleles (*ae-B4, ae-EMS1, ae-EMS3, ae-il, ae-M1,* and *ae-ref*) were used in this study. The W64A backcross conversions of the *amylose-extender* alleles were a gift from Dr. Douglas Garwood, The Pennsylvania State University, State College, Pennsylvania. Alleles were originally obtained from R. Bear (*ae-B4*), M. Zuber (*ae-M1*), R. Briggs (*ae-EMS1, ae-EMS3*), and R. Creech (*ae-il*). Small samples at the *ae* alleles are presently available from one of the authors (C.D.B.). The *ae-EMS1* and *ae-EMS3* alleles were derived from stocks after EMS mutagenesis; *ae-M1, ae-B4,* and *ae-ref* from spontaneous mutational events; and *ae-il* from stocks containing the *Ac-Ds*-controlling elements. The *ae-ref* allele (previously identified by Kramer *et al.*, 1956) was used in all previous studies and was included in the present study as the basis of comparison for the different alleles. The *ae* plants were field grown in 1980 and 1981 at Rutgers University Horticultural Farms, New Brunswick, New Jersey. Homozygous genotypes were produced by self- or sibling pollination. The ears were harvested 22 days after pollination, quick-frozen on dry ice, and stored at −80°C until used.

Starch branching enzymes and starch synthases from maize kernels were purified as previously described (Boyer and Preiss, 1981). The procedure consisted of homogenization, centrifugation, precipitation with 40% ammonium sulfate, and DEAE-cellulose chromatography. All steps were performed

at 0 to 4°C. The multiple forms of branching enzyme were located on the DEAE-cellulose profile based on the location of primed and citrate-stimulated starch synthases in the elution profile. Primed and citrate-stimulated starch synthases were measured by the incorporation of glucose from ADP-[^{14}C]glucose into methanol-insoluble glucan according to Hawker *et al.* (1974). Branching enzyme activity was measured by two separate assay procedures. *Assay A* measured branching enzyme activity by the amount of stimulation of α-1,4-glucan formation form [^{14}C]glucose-1-phosphate catalyzed by phosphorylase *a* (Boyer and Preiss, 1978a). *Assay B* measured branching enzyme activity by the decrease in absorbance at 660 nm of the amylose–iodine complex (Boyer and Preiss, 1978a). Protein levels were determined by the procedure of Lowry *et al.* (1951) using bovine serum albumin as the standard. Branching enzyme activity levels were corrected for enzyme loss during purification based on the activity measured in the initial extracts as described by Hedman and Boyer (1982).

RESULTS AND DISCUSSION

One or two purifications of enzymes from kernels homozygous for each of the *ae* alleles were performed using approximately 275 kernels from one or two ears. After DEAE-cellulose chromatography, 35 to 62% of the total branching enzyme was recovered, while 20.8 and 49.0% of the total activity was recovered for the primed and citrate-stimulated starch synthases, respectively.

Branching enzyme I was observed to elute in the buffer wash, while branching enzyme IIa was adsorbed onto the DEAE-cellulose and coeluted with the primed starch synthase after the application of a 0–0.4 M potassium chloride salt gradient to the column. Typical DEAE-cellulose profiles are shown in Fig. 1. Branching enzymes I, IIb, and IIa eluted in column fractions 10–30, 45–60, and 61–100, respectively. Characteristic absence of branching enzyme IIb activity was observed in extracts from all recessive genotypes (Fig. 1, Table I). The detection of small amounts of branching enzyme activity associated with the citrate-stimulated starch synthase fractions in some purifications was most likely the presence of branching enzyme IIa and was well within cross-contamination values determined previously (Boyer and Preiss, 1978a).

The activity of branching enzymes I and IIa was found to remain constant in extracts from all genotypes (Table I). The ratio of branching enzyme activity measured by two assays (Assay A, Assay B) did not vary with different *ae* mutants as well (Table I). The ratios of activities as measured by the two methods are characteristic for different branching enzymes and are consistent with previous studies (Boyer and Preiss, 1978a; Hedman and

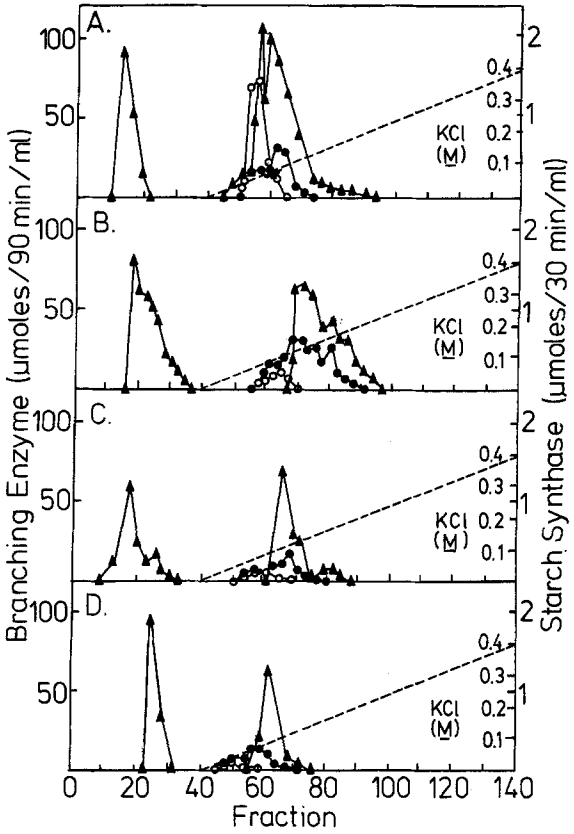

Fig. 1. DEAE-cellulose profiles of starch synthases and branching enzymes from maize kernels homozygous for differing *amylose-extender* (*ae*) alleles—citrate-stimulated starch synthase (○); primed starch synthase (●); and branching enzyme (▲); the dashed line indicates the position of the KCl gradient. (A) *Ae;* (B) *ae-ref;* (C) *ae-B4;* (D) *ae-EMS3.*

Boyer, 1982). Likewise, the relative activity of primed and citrate-stimulated starch synthases as measured with different primers are characteristic for the two enzymes. These primer specificities were unaffected by allelic substitution at the *ae* locus (data not shown).

These studies of *ae* alleles derived from different sources clearly show that all lack branching enzyme IIb activity. These results support the hypothesis of Hedman and Boyer (1982) that the recessive *ae* allele is a null allele for branching enzyme IIb. Investigation of the levels and properties of the branching enzymes and starch synthases has revealed that the *amylose-extender* locus affected solely the levels of branching enzyme IIb in each of the

Table I. Activity of the Branching Enzyme Fractions Isolated from Maize Kernels with Endosperm Homozygous for Differing *ae* Alleles

ae allele	Branching enzyme activity[a] (μmol/min/kernel)			Ratio of activity (Assay A/Assay B)[b]		
	I	IIb	IIa	I	IIb	IIa
Ae	1.04	1.70	0.85	24	270	474
ae-ref	1.24	0.00	1.04	41	ND[c]	373
ae-il	1.06	0.00	0.67	59	ND	623
ae-EMS1	1.02	0.07	0.50	24	ND	260
ae-EMS3	0.84	0.05	0.96	25	ND	414
ae-M1	0.61	0.15	0.53	46	ND	219
ae-B4	0.89	0.00	1.02	28	ND	382

[a] Measured by Assay A: stimulation of 1,4-glucan formation from [^{14}C]glucose-1-phosphate catalyzed by phosphorylase *a*.
[b] Ratio of activity (Assay A/Assay B) (stimulation of 1,4-glucan formation from [^{14}C]glucose-1-phosphate catalyzed by phosphorylase *a*/decrease in absorbance at 660 nm of amylose iodine complex).
[c] Not determined.

independently derived *ae* mutants examined. The *ae* mutants showed no branching enzyme IIb activity, while the levels and properties of branching enzyme I and IIa remained unchanged. In addition, the primed and citrate-stimulated starch synthases were unaffected. Previous characterization of the endosperm starches from kernels homozygous for different *ae* alleles showed little differences in the properties of the different starch fractions (Garwood *et al.*, 1976). These results are in agreement with the proposal that *amylose-extender* is the structural locus for branching enzyme IIb. If, in fact, a recessive *ae* allele is a null allele for branching enzyme IIb, then no functional branching enzyme would be produced. This theory is supported by the research presented here, in that no branching enzyme IIb activity was detected in extracts from any of the *ae* mutants examined. However, the possibility of *ae* mutants with only partially active branching enzyme IIb cannot be ruled out at this time. As selection of *ae* mutants has been based on kernel phenotype, mutants with partially active branching enzyme may not be identified because kernel phenotypes are unaffected. The detection of mutant kernels may require branching enzyme activity to be below a minimum threshold level.

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