

# Evidence for Independent Genetic Control of the Multiple Forms of Maize Endosperm Branching Enzymes and Starch Synthases<sup>1, 2</sup>

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CHARLES D. BOYER<sup>3</sup> AND JACK PREISS

Department of Biochemistry and Biophysics, University of California, Davis, California 95616

## ABSTRACT

Soluble starch synthase and starch-branching enzymes in extracts from kernels of four maize genotypes were compared. Extracts from normal (nonmutant) maize were found to contain two starch synthases and three branching enzyme fractions. The different fractions could be distinguished by chromatographic properties and kinetic properties under various assay conditions. Kernels homozygous for the recessive *amylose-extender* (*ae*) allele were missing branching enzyme IIb. In addition, the citrate-stimulated activity of starch synthase I was reduced. This activity could be regenerated by the addition of branching enzyme to this fraction. No other starch synthase fractions were different from normal enzymes. Extracts from kernels homozygous for the recessive *dull* (*du*) allele were found to contain lower branching enzyme IIa and starch synthase II activities. Other fractions were not different from the normal enzymes. Analysis of extracts from kernels of the double mutant *ae du* indicated that the two mutants act independently. Branching enzyme IIb was absent and the citrate-stimulated reaction of starch synthase I was reduced but could be regenerated by the addition of branching enzyme (*ae* properties) and both branching enzyme IIa and starch synthase II were greatly reduced (*du* properties). Starch from *ae* and *du* endosperms contains higher amylose (66 and 42%, respectively) than normal endosperm (26%). In addition, the amylopectin fraction of *ae* starch is less highly branched than amylopectin from normal or *du* starch. The above observations suggest that the alterations of the starch may be accounted for by changes in the soluble synthase and branching enzyme fractions.

The formation of the glucoside linkages of starch is believed to proceed by two reactions. ADP-glucose is utilized as a glucosyl donor for formation of new  $\alpha$ -1,4-glucosyl linkages in a reaction catalyzed by starch synthase (EC 2.4.1.21). The  $\alpha$ -1,6 linkages are then introduced into the  $\alpha$ -glucan by transfer of a part of the growing  $\alpha$ -1,4-polyglucose chain to the hydroxyl group of 6-carbon of a glucosyl unit of either another chain or to another part of the same glucan chain (EC 2.4.1.18). Analyses of spinach leaves (12, 19), potatoes (11), and several maize genotypes of differing endosperm type (3, 4, 18) have shown the presence of multiple forms of starch synthases and branching enzymes.

Full understanding of the role of multiple enzyme forms in the biosynthesis of the starch granule and its components, amylose

and amylopectin, can be accomplished only by purifying and characterizing the different enzymes involved. Present efforts have involved characterizing the branching enzyme and starch synthases found in maize endosperm. This report is concerned with the presence of multiple forms of both starch synthases and branching enzymes in maize endosperm and the absence of some of the enzyme forms in two nonallelic mutants affecting the development of the maize endosperm, *amylose-extender*, *ae*, and *dull*, *du*. The availability of maize mutants, detected because of modified endosperm cell and starch granule development due to modification to chemical structure of starch, should provide model systems for determining the route of biosynthesis of starch. The basis of the genetic modifications must eventually be explained at the level of the starch biosynthetic enzymes.

## MATERIALS AND METHODS

ADP-[<sup>14</sup>C]glucose was prepared as previously described (22). The specific radioactivity used in the experiments was usually 500 cpm/ $\mu$ mol. Rabbit liver glycogen type III and oyster glycogen were purchased from Sigma; maize amylopectin and potato amylose came from Calbiochem. *Escherichia coli* glycogen was prepared as described (23). All other reagents were obtained commercially at the highest available purity.

The maize inbred line W64A and the *ae* backcross conversion of W64A were grown in the field in 1977. The *du* backcross conversion of W64A and the W64A double mutant *ae du* were greenhouse grown in 1978. All plants were self- or sib-pollinated. Ears were harvested 22 days after pollination, frozen in Dry Ice within 5 min after removal from the plant, and stored at  $-20^{\circ}\text{C}$  until used.

**Starch Synthase Assay.** Incorporation of glucose into primer.

**Assay A.** Reaction mixtures were those previously described (12). Total reaction volumes were 0.2 ml and all final primer concentrations were 5 mg/ml. After incubation at 30  $^{\circ}\text{C}$ , [<sup>14</sup>C]-glucose incorporated from ADP-[<sup>14</sup>C]glucose into a methanol-insoluble product was determined by the method of Ghosh and Preiss (8). One unit of enzyme is defined as 1  $\mu$ mol of glucose transferred per min.

**Assay B: citrate-stimulated.** Incorporation of [<sup>14</sup>C]glucose from ADP-[<sup>14</sup>C]glucose into a methanol-insoluble product in the absence of primer was followed as described (12). Reaction volumes were 0.2 ml. Reactions were incubated at 30  $^{\circ}\text{C}$  and heat-killed (1 min at 100  $^{\circ}\text{C}$ ), and then carrier glycogen (1 mg) was added and counted as described (12).

**Branching Enzyme Assay.** Phosphorylase stimulation.

**Assay A.** The branching enzyme assay is based on the ability of branching enzyme to stimulate the incorporation of glucose from [<sup>14</sup>C]glucose-1-P (50 cpm/ $\mu$ mol) into endogenous glucan by rabbit liver phosphorylase. The assay was done as previously described (12). One unit of enzyme is defined as 1  $\mu$ mol of glucose incorporated per min.

**Assay B. Amylose Branching.** The fall in *A* at 660 nm of the

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<sup>3</sup> Present address: Department of Horticulture and Forestry, Cook College, Rutgers University, New Brunswick, NJ 08903.

amylose-iodine complex was determined as described by Boyer and Preiss (4). One unit of enzyme is defined as the decrease in *A* of the amylose-iodine complex of 1.0 per min.

**Protein Determination.** Protein in all fractions was determined by the method of Lowry *et al.* (15).

**Enzyme Purification.** Enzymes have been purified from as little as 20 g and as much as 500 g of tissue with similar results. All procedures were carried out at 4 C. Tissue was initially homogenized in a small amount of cold 50 mM Tris-acetate buffer (pH 7.5) containing 10 mM EDTA and 2.5 mM dithioerythritol. The homogenate was filtered through several layers of cheesecloth, and homogenization and filtering were repeated as necessary. The final volume of the crude extract was 1 to 2 ml per g of tissue. The crude homogenate was centrifuged at 45,000g for 30 min. The supernatant fraction was made 40% saturated with solid ammonium sulfate, and the precipitated protein was pelleted by centrifugation at 10,000g for 20 min.

The pelleted protein was dissolved in a small amount of the Tris-acetate buffer system above containing 10% sucrose, then dialyzed against 1.5 to 2.0 liters of the same buffer for 8 to 12 h. The dialyzed solution was loaded onto a DEAE-cellulose column equilibrated with 50 mM Tris-acetate (pH 7.5), 10 mM EDTA, 2.5 mM dithioerythritol, and 10% sucrose solution. The DEAE-cellulose columns contained 1 ml of resin bed volume per 3 to 5 mg of total protein in the dialyzed fractions. After loading, the column was washed with at least 2 bed volumes of the equilibration buffer system, followed by a 0- to 0.4-M KCl gradient in the buffer system (10 to 12 times the bed resin volume). Fraction volumes were adjusted so that 90 to 100 fractions were collected during the gradient. Peak fractions were pooled, concentrated, and dialyzed as described previously (4).

**Starch Purification and Characterization.** Starch granules were extracted from dissected endosperm tissue by repeated extraction of contaminating protein with toluene as previously described (6). Purified starch granules were dispersed in 90% dimethylsulfoxide at a concentration of 10 mg/ml. The dispersed starch granules were precipitated with 3 volumes of 7% methanol-1% KCl solution and redissolved in 90% dimethylsulfoxide at the same concentration. Aliquots (0.5 ml) of the dispersed starch were precipitated as above and redissolved in 0.5 ml 0.01 N NaOH containing 0.02% sodium azide. The aliquot was chromatographed on a Bio-Gel A-5.0m column (1.5 × 30 cm) equilibrated with 0.01 N NaOH containing 0.02% sodium azide. Fractions (1 ml) were collected, neutralized with 1 N HCl, and assayed with iodine (14).

Amylose content was estimated from column fractions. Column fractions which had higher *A* of the iodine-starch complex at 540 nm than at 660 nm were pooled as amylopectin; fractions with higher *A* at 660 nm were pooled as amylose. Amylose contents of the starches were also determined by the blue value procedure (24). Carbohydrate was determined in both procedures by the anthrone-sulfuric acid test (1).

## RESULTS AND DISCUSSION

**Properties of Maize Endosperm Starch Synthases.** It had been previously reported that two major forms of starch synthase could be separated by DEAE-cellulose chromatography from extracts of maize endosperm (3, 4, 18), potato (11), and spinach leaf (12, 19). The two fractions in maize could be distinguished from each other with respect to their reaction rates for different primers. A very distinct difference is that one fraction in each of the plant extracts is able to catalyze the synthesis of an  $\alpha$ -1,4-glucan with the slight amount of endogenous primer associated with the enzyme in the presence of 0.5 M citrate. DEAE-cellulose chromatography of an ammonium sulfate fraction of dent maize endosperm extract resolved starch synthase activity into two fractions (3, 4). Fraction I had higher activity with glycogen than with amylopectin (Table

I); the reverse was true with fraction II. In the presence of 0.5 M citrate, fraction I is able to catalyze transfer of glucose from ADP glucose into an alcohol-insoluble material that can be degraded by  $\alpha$ -amylase. Fraction II, however, cannot. Other polyvalent salts, such as DL-malate, EDTA, and sulfate can replace citrate in stimulating this reaction. The reaction in the presence of 0.5 M citrate and the endogenous primer associated with the starch synthase will be referred to as the citrate-stimulated reaction.

Table I summarizes the properties of maize starch synthases I and II purified through the DEAE-cellulose chromatographic step. Both enzymes had similar  $K_m$  values for ADPglucose (0.1 mM). Fraction I had about four times more activity with rabbit liver glycogen as primer than with amylopectin. Other glycogens were also more active than amylopectin. However, fraction II showed greater activity with amylopectin as primer than with the various glycogen preparations.

**Properties of Maize Endosperm Branching Enzymes.** Fractionation of an  $(\text{NH}_4)_2\text{SO}_4$  fraction of maize endosperm extract on DEAE-cellulose showed three different fractions of branching enzyme activity. Branching enzyme I, the first fraction, is seen in the pass-through effluent. Fractions IIa and IIb can also be separated by hydrophobic chromatography on a 4-aminobutyl-Sepharose column, and all isozymes are purified further by gel filtration on a Bio-Gel A-1.5m column (4).

The properties of the three branching enzyme fractions after purification have been summarized and have been shown to be different for each enzyme form when assayed by the procedures (4) indicated in the "Materials and Methods."

**Distribution of Amylose and Amylopectin in Mutant Maize Endosperms.** It appears that there are distinct forms of starch-branching enzyme activities in maize endosperm. To ascertain their function as well as the function of the multiple forms of the starch synthases, it was decided to study some nonallelic mutants of maize. Two of these mutants, *ae* and *du*, qualitatively alter the distribution of amylose and amylopectin in starch (Fig. 1). Starch from homozygous *ae* endosperms, in addition to containing a higher proportion of linear amylose, contains a fraction, designated anomalous amylopectin, that has fewer branch points than does normal amylopectin (*i.e.* greater average unit chain length), a decreased solubility in  $\text{H}_2\text{O}$ , and an increased affinity for  $\text{I}_2$  (17). The difference in amylopectin from *ae* endosperm can be illustrated by the observation that the amylose content determined from the column was 60%, but the amylose content estimated from the blue value was 66%. The blue value method, which is based on the *A* of the iodine-starch complex at 615 nm, overestimates the amylose content due to the greater affinity of *ae* amylopectin for iodine. Amylose content of normal was in fact, higher when determined by the column method (30%) than when determined by the blue value method (26%). A higher than normal proportion of amylose was also contained in *du* endosperms (Fig. 1). However, the amylopectin and amylose components did not appear significantly different in this mutant. The amylose content

Table I. Properties of Maize Starch Synthases I and II  
The % activity of amylopectin was arbitrarily set at 100%.

Primers	Activity of	
	Synthase I	Synthase II
	%	
Amylopectin	100	100
Rabbit liver glycogen	431	79
Oyster glycogen	150	29
<i>E. coli</i> glycogen	242	66

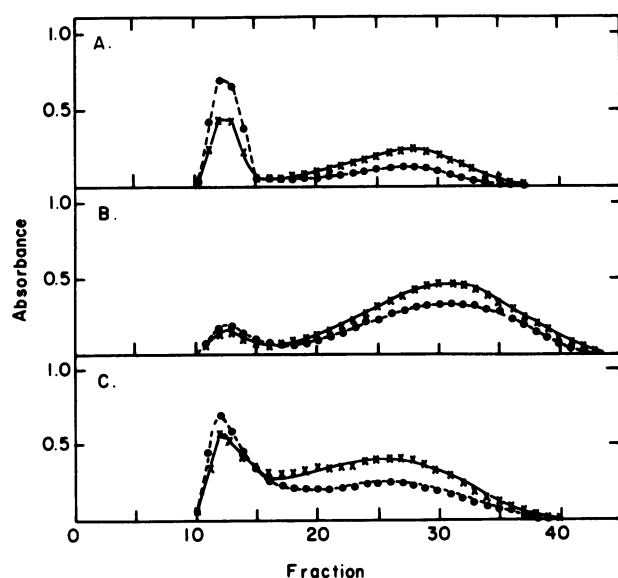


FIG. 1. Chromatography of 90% dimethylsulfoxide-dispersed starches from normal maize endosperm (A), *ae* maize endosperm (B), and *du* maize endosperm (C). Fractions were assayed by the addition of iodine reagent. X, A of  $I_2$ -starch complex at 660 nm; ●, A of  $I_2$ -starch complex at 540 nm. The amylopectin content (first peak) and amylose content (second broad peak) were determined as indicated in "Material and Methods."

of *du* starch was estimated at 42% and 44% by the blue value and the column methods, respectively. The altered natures of the starches from these two mutants suggest that these mutants affect either branching enzyme activity or the ratio of starch synthase and branching enzyme.

**Starch Synthases and Branching Enzymes of Mutant Maize Endosperms.** The DEAE-cellulose chromatographic pattern of *ae* endosperm extracts has been compared with normal endosperm extracts (3). Fraction I activity is equivalent to that found in normal maize extracts. The kinetic properties of maize branching enzyme I of the normal maize and *ae* have been compared; they appear to be similar with respect to their ratios of activities in the branching enzyme assays A and B (about 30:60). The  $K_m$  for amylose in the  $I_2$ -complex assay (assay B) is the same for both enzymes (160  $\mu$ g/ml), and the mol wt values (89,000) obtained by disc gel electrophoresis and gel filtration are similar.

In contrast, there is an 80% reduction of branching enzyme activity in the total fraction II peak in *ae* extracts (3). The levels of primed starch synthase I and II activities in the *ae* crude extracts, however, are roughly equivalent to the activities observed in normal maize extracts. Most, if not all, of the branching enzyme activity associated with starch synthase I fractions is missing. Moreover, the citrate-stimulated activity associated with starch synthase I is considerably reduced. This citrate-stimulated activity is restored by addition of any of the three branching enzymes from normal maize (I, IIa, or IIb). The stimulation is about 3.5- to 4-fold (Table II), bringing the total activity up to the level observed for normal maize preparations. Branching enzymes obtained from other genotype maize extracts are also effective (unpublished results). Citrate-stimulated starch synthase I activity from normal maize is not stimulated by addition of branching enzyme because the branching enzyme activity is already in excess. These data indicate that branching enzyme stimulates the starch synthase I activity in the presence of 0.5 M citrate when primer concentrations are limiting and are consistent with previous observations with spinach leaf starch synthase (12) and with *Escherichia coli* B glycogen synthase (2, 7, 13). Characterization of the

Table II. Stimulation of the Citrate-Stimulated Reaction Catalyzed by Starch Synthase I by Branching Enzyme from Different Maize Genotypes

Activity in absence of branching is arbitrarily set at 100%. The specific activities of the starch synthase fractions used were comparable for all fractions and were 0.013 to 0.02  $\mu$ mol of glucose transferred per min at 30 C.

Starch Synthase I Source <sup>a</sup>	Activity	
	–Branching enzyme	+Branching enzyme <sup>b</sup>
	%	
Normal	100	100 $\pm$ 0.05
<i>Amylose extender</i>	100	350 $\pm$ 25
<i>Amylose extender, dull</i>	100	380 $\pm$ 25

<sup>a</sup> Pooled fractions from DEAE-cellulose chromatography.

<sup>b</sup> Branching enzyme I fractions 0.05 to 0.20 unit/reaction mixture purified from normal, *ae*, *du*, and *ae du* were used. The ratio of branching enzyme units to starch synthase units ranges from 50:1 to 200:1 (5).

citrate-stimulated reaction of starch synthase I from *ae* has been reported (5, 21).

Kinetic studies with *ae* starch synthase I and II showed similar  $K_m$  values for ADP glucose that are observed for the normal starch synthases (0.1 mM). The relative levels of activities observed for various primers with *ae* starch synthases I and II were the same as listed in Table I for the normal synthases I and II. Thus, the mutation in *ae* maize endosperm appeared to affect the level of branching enzyme II activity, particularly fraction IIb. The level of starch synthase activities and the kinetic studies indicate that they are not affected by the *ae* mutation. The demonstration of loss of branching enzyme activity in *ae* extracts is consistent with the greater percentage of amylose and abnormally higher average chain length amylopectin found in the *ae* endosperm (3). Furthermore, purification of branching enzymes and starch synthases from kernels with endosperm genotypes with 0, 1, 2, and 3 doses of the *AE* allele have shown a near linear proportion of branching enzyme IIb activity with the number of normal *AE* alleles present (Hedman and Boyer, unpublished results).

Figure 2 compares the DEAE-cellulose chromatography of starch synthases and branching enzymes from *du* endosperm extracts with normal endosperm extracts. Whereas the levels of branching enzyme fraction I and starch synthase fraction I are comparable in the extracts, there appears to be a significant diminution of starch synthase II activity (60%) and a small decrease of the branching enzyme activity associated with the starch synthase II fractions. The citrate-stimulated activity of *du* starch synthase I is similar to that observed for normal starch synthase I, since the level of branching enzyme activity in those fractions is similar to those seen in normal maize extracts. This low level of starch synthase II activity is also observed in extracts of the endosperm of the double mutant, *ae du*. Figure 2 also compares the DEAE-cellulose chromatography of the starch synthases and branching enzymes of the double mutant *ae du* endosperm with the normal endosperm enzymes. Branching enzyme fractions IIa and IIb and starch synthase fraction II in the *ae du* mutant are considerably reduced (>80%). Starch synthase fraction I primed activity and branching enzyme fraction I activities are equivalent to that observed for normal endosperm. The citrate-stimulated activity of starch synthase I is low in the double mutant, but, as in *ae*, this is due to the absence of branching enzyme activity in the *ae du* starch synthase I fractions. Addition of branching enzyme in excess restored the citrate-stimulated activity of starch synthase I to normal levels (Table II). The expression of *ae* and *du* are unaffected by the allelic state at the other locus.

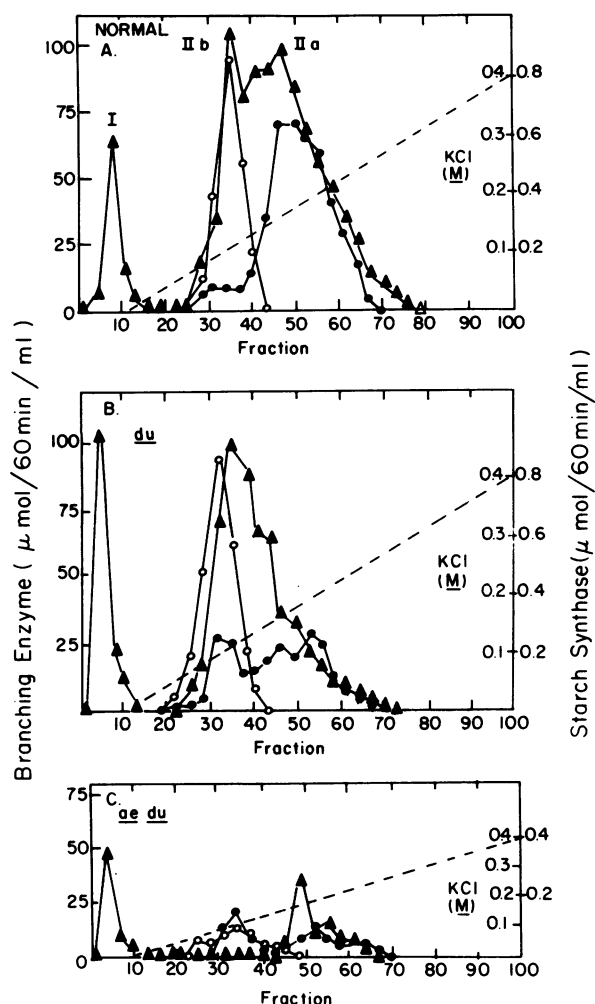


FIG. 2. Chromatography of maize branching enzyme and starch synthases on DEAE-cellulose.  $\Delta$ , Branching enzyme activity;  $\circ$ , citrate-stimulated starch synthase activity;  $\bullet$ , primed starch synthase activity as described in "Materials and Methods." ---, Linear KCl gradient. A, chromatography of normal extract as previously described (4). B, chromatography of extract from *du* kernels (23 g). Protein (92 mg) was loaded onto a 1.5  $\times$  23-cm column. The column was washed with 120 ml buffer followed by a 1,000-ml 0-0.4-M KCl gradient. C, Chromatography of extract from *ae du* kernels (25 g). Protein (131 mg) was loaded onto a 1.5  $\times$  27-cm column. The column was washed with 150 ml buffer followed by a 1,000-ml 0-0.4-M KCl gradient.

The data suggest that the *du* mutation may affect the starch synthase II activity. There also appears to be a lowering of the branching enzyme IIa activity associated with this genotype. Since both starch synthase and branching enzyme activity may be affected by the *du* mutation and since the *du* starch granule fractions of amylopectin and amylose have not been fully characterized, it is premature to relate the effects of the *du* mutation on enzyme levels to the effect on the structure of starch. The *du* mutation appears to be the first maize mutation known to affect the level of soluble starch synthase activity. This finding strongly indicates the importance of the starch synthase in synthesizing a normal starch granule. Because two enzyme activities are diminished by the *du* mutation, it is quite possible that a regulatory gene rather than a structural gene has been affected.

One may speculate why three different branching enzymes and two starch synthases are required for synthesis of amylopectin in maize or in other plants. There are numerous reports of multiple

starch synthases and branching enzymes in various plant extracts (3, 4, 10-12, 20), thus suggesting that they are distinct, functional activities. The answer may be suggested by data reported from the laboratory of Whelan *et al.* (9, 16) on the structure of glycogen and amylopectin. When amylopectin, glycogen, or phytoglycogen are debranched with the appropriate debranching enzyme and the resulting products fractionated by gel filtration, glycogen exhibits a broad but single peak containing oligosaccharide chains from 3 to 25 glucosyl units long. Phytoglycogen chain lengths are less uniform. However, amylopectin chains fall into two groups of varying chain lengths between 12 and 42 units long. These results have been confirmed in our laboratory (C. D. Boyer and J. Preiss, unpublished results). Amylopectin appears to be asymmetric in oligosaccharide chain lengths when compared to glycogen. It is quite possible that the isozymic forms of starch synthase and branching enzyme having different specificities for elongation and for branching may interact to synthesize the different lengths of chains found in amylopectin. Thus, starch synthase I had branching enzyme IIb and starch synthase II and branching enzyme IIa may behave as two different enzyme complexes with different specificities that, upon interaction, give rise to the asymmetric amylopectin product. Further studies are required to establish more precisely the relationships of the multiple forms of branching enzymes and starch synthases and the asymmetric nature of the chain lengths of amylopectin.

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