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MULTIPLE FORMS OF (1 → 4)-α-D-GLUCAN, (1 → 4)-α-D-GLUCAN-6-GLYCOSYL TRANSFERASE FROM DEVELOPING *Zea mays* L. KERNELS*†

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ABSTRACT

Two major forms of branching enzyme from developing kernels of maize have been detected after DEAE-cellulose chromatography. Branching-enzyme I, which contained 24% of the activity based on a phosphorylase-stimulation assay, but 74% of the activity based on the branching of amylose as monitored by change in spectra of the iodine–glucan complex, eluted with the column wash and was unassociated with starch-synthase activity. Branching-enzyme II was bound to DEAE-cellulose and was coeluted with both primed and unprimed starch-synthase activities. Both fractions were further purified by chromatography on aminoalkyl-Sepharose columns. Single peaks were observed for both fractions by gel filtration on BioGel $A_{1.5m}$ columns and native molecular weights were estimated at 70,000–90,000 for both enzymes. Subunit molecular weights of branching-enzymes I and II were estimated by dodecyl sodium sulfate-gel electrophoresis at 89,000 and 80,000, respectively. Thus both enzymes are primarily monomeric. Branching-enzymes I and II could be distinguished by chromatography on DEAE-cellulose or 4-aminobutyl-Sepharose, and by disc-gel electrophoresis with activity staining. Branching-enzyme I had a lower ratio of activity (phosphorylase stimulation–amylose branching; based on enzyme units). The ratio varied from 30–60 as compared to about 300–500 for branching-enzyme II. Likewise, branching-enzyme I had a lower K_m value for amylose than branching-enzyme II, the values being 160 and 500 μg/ml, respectively. Both enzymes could introduce further branches into amylopectin, as decreases in the overall absorption and wavelength maxima of the iodine complexes were observed. Combined action of the branching enzymes and rabbit-muscle phosphorylase *a* (12:1 ratio based on enzyme units) resulted in similar patterns of incorporation of D-glucose into the growing α-D-glucan and the synthesis of high molecular-weight polymers. However, the α-D-glucans differed, as shown by spectra of iodine complexes and average unit-chain length. Branching-enzyme II was separated into two fractions (IIa and IIb) by

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chromatography on 4-aminobutyl-Sepharose. These fractions differed only in the branching of amylopectin, fraction IIb being more active than IIa.

INTRODUCTION

The synthesis of the (1 → 6)-α-D-glucosidic linkages of the amylopectin fraction of starch is considered to be catalyzed by (1 → 4)-α-D-glucan, (1 → 4)-α-D-glucan-6-glycosyl transferase (EC 2.4.1.18, plant branching-enzyme). Although the enzyme has been shown to act by the transfer of a chain fragment, removed from a donor by breakage of a (1 → 4) bond, to an acceptor (which may or may not be the original donor) with the formation of a (1 → 6) bond[1], this mechanism involves no net synthesis of D-glucan. This lack of net synthesis has made the action of branching enzymes difficult to assay quantitatively. Branching-enzyme activity is most frequently assayed by monitoring the branching of amylose as measured by the decrease in absorbance of the amylose–iodine complex resulting from the branching[1]. Although this assay procedure is relatively simple, it has the disadvantages of being insensitive, hard to interpret, and not quantitative. Furthermore, despite considerable effort by many researchers, it has been impossible to demonstrate the synthesis of amylopectin by the simple branching of amylose, even extensively[1]. Thus this assay and reaction may not reflect any meaningful physiological process.

Alternatively, branching enzymes may be assayed by utilization of the stimulation, caused by the addition of branching enzyme[2,3], of unprimed synthesis of α-D-glucan from α-D-glucosyl phosphate by phosphorylase *a*. The stimulation results from an increase with branching in the number of nonreducing ends in the growing α-D-glucan, and thus an increase in the number of sites for further synthesis of (1 → 4)-α-D-glucan chains. This assay has the advantages of being sensitive (at least 50–100 fold greater than the amylose-branching assay) and being semiquantitative, with synthesis of α-D-glucan being measured. Although both assays are used to measure branching activity, two different reactions are occurring. When amylose is branched, a substrate of a high degree of polymerization (300–1000 glucose residues) is used and the initial reaction constitutes the addition of a few branches per molecule. By contrast, in the coordinate assay-system of phosphorylase *a* and branching enzyme, the initial substrate is a small, growing, α-D-glucan chain that eventually reaches a degree of polymerization sufficient to be branched. Subsequently, the substrate is branched, but the nonreducing ends are still growing, continually presenting long, outer, linear (1 → 4) regions for further branching. This elongation and branching of the α-D-glucan chain may reflect the true physiological process of amylopectin synthesis when starch synthase is the elongating enzyme. By using both assay systems for branching enzymes, we have been able to identify multiple forms of branching enzyme in developing kernels of maize. The following reports the partial purification and initial characterization of the multiple forms found.

EXPERIMENTAL

Materials. — ADP-D-[^{14}C]glucose was prepared enzymically from D-[U-^{14}C]-glucose[4]. Maize amylopectin (amylose free) and potato amylose (amylopectin free) were purchased from Calbiochem, Los Angeles, CA; rabbit-muscle phosphorylase *a* from Sigma, St. Louis, MO; and α-D-[^{14}C]glucosyl phosphate from Amersham–Searle Corp., Arlington Heights, IL. Isoamylase isolated from *Cytophaga*[5] was a gift from Dr. Z. Gunja-Smith of the Department of Biochemistry, University of Miami School of Medicine, Miami, FL. Aminoalkyl-Sepharose columns were prepared as described by Shaltiel and Er-El[6]. All other reagents were of highest purity. The dent maize inbred W64A was field-grown in 1976 and plants were self or sib pollinated. Ears were harvested 22 days after pollination, quick frozen on Dry Ice, and stored at −15° until used.

Assay of maize-branching enzymes. — *Assay A.* The basis of the assay is the stimulation by branching enzyme of the synthesis of α-D-glucan from α-D-glucosyl phosphate catalyzed by rabbit muscle phosphorylase[2,3]. The mixture contained, in a volume of 0.2 ml, 0.1M sodium citrate (pH 7.0), 1mM AMP, 50mM α-D-[^{14}C]glucosyl phosphate (5.0×10^4 c.p.m./μmol), 40 μg of crystalline, rabbit-muscle phosphorylase *a*, and branching enzyme. The reaction was initiated by addition of α-D-glucosyl phosphate and the incubation was at 30°. Aliquots were taken at 60, 90 and 120 min and incorporation of label into the glucan was assayed as previously described[4]. One unit of enzyme activity is defined as 1 μmol of D-glucose incorporation into D-glucan per min under the foregoing conditions. Controls containing no branching enzyme, or heat-denatured (1 min at 100°) branching enzyme, incorporated less than 20 nmol of D-glucose into the D-glucan fraction. Control values were subtracted from those obtained from mixtures containing active branching-enzyme. Column fractions were assayed by halving the reaction volume and incubating for 90 min.

Decrease of absorption of the amylose–iodine complex. — *Assay B.* Mixtures containing 100 μg of amylose, 10 μmol of sodium citrate (pH 7.0), and branching enzyme per 0.1 ml volume (in a total volume of 0.35 ml) were incubated at 30°, and 50-μl aliquots were sampled at timed intervals. Water (0.35 ml) and 2.6 ml of iodine reagent were added. Iodine reagent was made daily from 0.5 ml of stock solution (0.26 g of I_2 and 2.6 g of potassium iodide in 10 ml of water) mixed with 0.5 ml of *M* hydrochloric acid, and diluted[7] to 130 ml. The reactions were monitored by the change in absorbance of the glucan–iodine complex over the range of 400–700 nm. One unit of activity is defined as a decrease in absorbance of 1.0 per min at 660 nm, measured as already described.

Protein determination. — Proteins were assayed by the method of Lowry *et al.*[8].

Assay for starch-synthase activity. — The incorporation of D-glucose into α-D-glucan from ADP-D-glucose under primed and unprimed conditions was measured by the procedure of Hawker *et al.*[4].

Assay for phosphorylase. — Phosphorylase was measured in the direction of

synthesis by using phytoglycogen isolated from a commercial sweet corn as primer[9].

Assay for alpha amylase and R-enzyme. — These enzymes were assayed as described by Drummond *et al.*[1].

Electrophoresis. — Disc-gel electrophoresis was performed on 6% poly(acrylamide) gels with the Ornstein–Davis Tris–glycine buffer system[10,11]. 1,4-Dithiothreitol (5 mM) was included in all buffers. In addition, the running gel was polymerized in the presence of amylose (0.3 mg/ml). Active bands were detected by incubation of the gels in 100 mM sodium citrate buffer, pH 7.0, containing 5mM DTE, followed by staining with the iodine reagent already described (Assay B). Gel electrophoresis in the presence of dodecyl sodium sulfate was performed in 9% poly(acrylamide) gels as described by Neville[12]. Various standard proteins were used in the estimation of the molecular weight of the branching-enzyme subunit. Protein bands in both systems were located by staining with Coomassie Blue[13].

Formation of branched polysaccharides by branching enzymes. — The specificity of the maize branching-enzymes were determined by assaying the decrease in absorbance of the glucan–iodine complex of amylose and amylopectin under the conditions already described (Assay B). Mixtures contained 5 units (Assay A) of branching enzyme per ml. The reactions were monitored by the change in absorbance of the glucan–iodine complex over the range of 400–700 nm. Polysaccharides were also formed by the coordinate action of phosphorylase *a* and the branching-enzyme fractions, singly and all combined. The conditions of Assay A were used. No primer was added, but is presumably present in the phosphorylase preparation. Phosphorylase was kept constant at 1.5 IU per ml. A ratio of branching-enzyme activity to chain-elongation enzyme activity of 12 (based on enzyme units, Assay A) was used. Reactions were monitored by incorporation of radioactive D-glucose in the growing α-D-glucan and the appearance of iodine-complexing glucan. All reactions were stopped after 2 h by boiling for 5 min and polysaccharides were isolated by precipitation with 75% methanol containing 1% of potassium chloride.

Debranching of polysaccharides and separation of linear (1 → 4)-α-D-glucans. — Branched polysaccharides (1–5 mg) were debranched at 37° with 1 unit of isoamylase (1 mg/ml) in digests containing 100mM sodium acetate buffer, pH 5.5. After incubations for 20–24 h, the enzymes were inactivated by heating for 10 min at 100°. The average degree of polymerization (d.p.) of the unit chains were determined by the ratio of total glucose equivalents versus reducing glucose equivalents[5]. Total glucose equivalents were determined by the anthrone–sulfuric method[14] and reducing equivalents by a copper reducing method[15].

Estimation of molecular weights of α-D-glucans. — Molecular weights of the α-D-glucan products were estimated by gel filtration on a column (1.5 × 30 cm) of Bio-Gel A_{50m}. Polysaccharides were eluted with 0.01M sodium hydroxide containing 0.02% sodium azide, and 1.0 ml fractions were collected. The column was calibrated with Blue Dextran (Pharmacia), amylopectin, and amylose. Total carbohydrate in fractions was determined by the anthrone–sulfuric method[14] or by liquid-scintillation spectrometry.

RESULTS

Extraction of the enzyme. — Kernels (185 g) were ground with a porcelain mortar and pestle in the presence of 100 ml of cold, 50mM Tris–acetate buffer (pH 7.5) containing 2.5mM dithiothreitol and 10mM EDTA. Pericarps and incompletely ground endosperm were collected by passing the homogenate through 4 layers of cheesecloth and the grinding was repeated twice more. After the third grinding, only translucent pericarps remained on the cheesecloth. The extracted solution constitutes the crude extract. All purification procedures were carried out at 0–4°.

Isolation of the 45,000 g-supernatant fraction. — The crude extract was centrifuged at 45,000*g* for 20 min to remove starch granules from the enzyme solution. No loss of activity was observed at this step (Table I).

Fractionation with ammonium sulfate. — The 45,000*g* supernatant was made 40% saturated by addition of crystalline ammonium sulfate. The mixture was stirred for 20 min and centrifuged at 16,000*g* for 20 min. The supernatant was discarded and the precipitate was dissolved in the minimal volume of 50mM Tris–acetate buffer (pH 7.5) containing 2.5mM 1,4-dithiothreitol, 10mM EDTA, and 5% sucrose, and dialyzed against 2 liters of the same buffer.

Chromatography on DEAE-cellulose. — The dialyzed solution from the preceding step was absorbed onto a column (2.5 × 23 cm) of DEAE-cellulose (Whatman DE-52) that had been equilibrated with 50mM Tris–acetate buffer (pH 7.5) containing 2.5mM 1,4-dithiothreitol, 10mM EDTA, and 5% sucrose. The column was washed with 2 resin-bed volumes, and a 2-liter gradient of 0–0.4M potassium chloride applied. Fractions of 20 ml were collected. Two peaks of branching activity were observed (Fig. 1). Fraction I eluted with the wash and Fraction II was absorbed onto the column and was coeluted in the gradient with both the unprimed and primed starch-synthase activities [ADP-D-glucose (1 → 4)-α-D-glucan, 4-α-D-glucosyl transferase]. Fractions 4–13 (Fraction I) and 29–66 (Fraction II) were pooled and concentrated by ultrafiltration. Table I summarizes the purification through DEAE-cellulose.

TABLE I

PURIFICATION OF MAIZE (1 → 4)-α-D-GLUCAN, (1 → 4)-α-D-GLUCAN-6-GLYCOSYL TRANSFERASES I AND II

Fraction	*Volume (ml)*	*Protein (mg)*	*Total activity*[a] *(units)*	*Specific activity*[a] *(units)*
1. Crude	333	3580	5410	1.51
2. 45,000 *g* supernatant	305	1107	5532	5.00
3. $(NH_4)_2SO_4$	70	424	4954	11.7
4. DEAE-cellulose				
Fraction I	42	117	891	7.6
Fraction II	23	89.3	2799	31.3

[a]Activity measured by Assay A as described in the text.

Fraction I contained 24% of the total activity recovered when assayed by phosphorylase *a* stimulation (Assay A). However, when the two fractions were assayed by the branching of amylose (Assay B), Fraction I contained 74% of the total activity recovered. Fractions I and II had ratios of activity in the two assay systems (Assay A/Assay B) of 56 and 490, respectively.

Further purification of maize branching-enzyme I. — Concentrated DEAE Fraction I was adsorbed onto a column (1.5 × 18 cm) of 5-aminopentyl–Sepharose equilibrated with 50mM Tris–acetate buffer (pH 7.5) containing 2.5mM 1,4-dithiothreitol and 10mM EDTA. The column was washed with 175 ml of buffer and the enzyme eluted with an 800-ml, linear gradient of 0–1.0M potassium chloride (Fig. 2A).

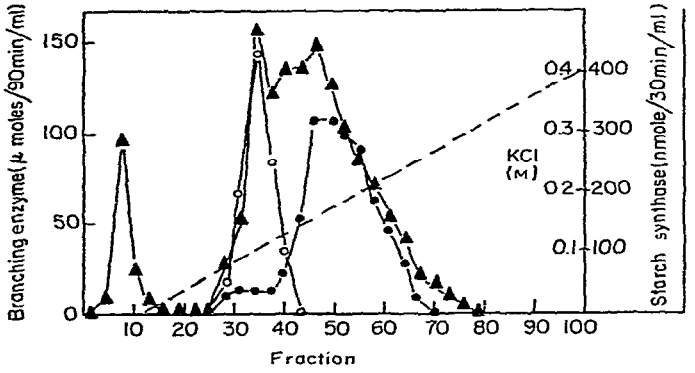

Fig. 1. Chromatography of maize branching-enzymes on DEAE-cellulose; branching-enzyme activity, Assay A (▲), unprimed starch-synthase activity (○), primed starch-synthase activity (●). The procedure is described in the text.

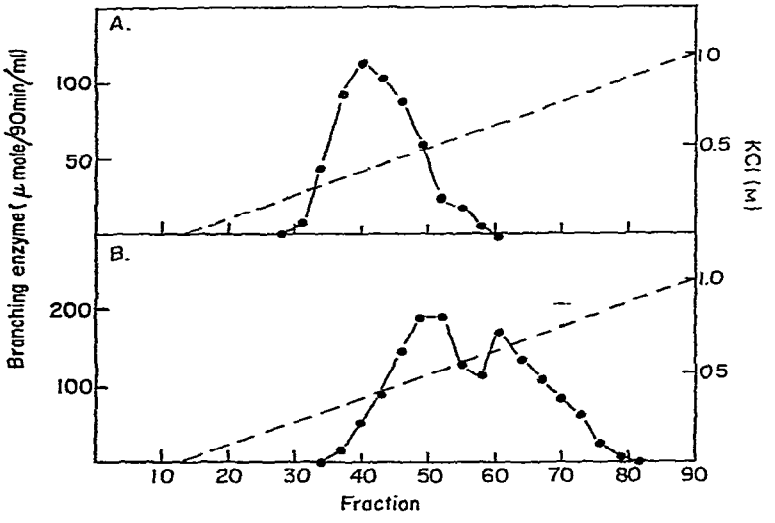

Fig. 2. Chromatography of maize branching-enzymes I and II on aminoalkyl-Sepharose columns. Branching enzymes were assayed by Assay A. A, Chromatography of branching-enzyme I on 5-aminopentyl-Sepharose; B, Chromatography of branching-enzyme II on 4-aminobutyl-Sepharose.

TABLE II

PURIFICATION OF MAIZE (1 → 4)-α-D-GLUCAN, (1 → 4)-α-D-GLUCAN-6-GLYCOSYL TRANSFERASE I

Fraction	*Volume (ml)*	*Protein (mg)*	*Total activity (units)*		*Specific activity (units/mg)*		*Ratio Assay A/Assay B*
			Assay A	*Assay B*	*Assay A*	*Assay B*	
DEAE-cellulose	42	117	891	16.05	7.61	0.137	55.5
5-Aminopentyl-Sepharose	6.3	23.4	489	9.25	20.9	0.395	52.9
Bio Gel $A_{1.5m}$	9.7	10.2	304	8.56	29.8	0.839	35.5

TABLE III

PURIFICATION OF MAIZE (1 → 4)-α-D-GLUCAN, (1 → 4)-α-D-GLUCAN-6-GLYCOSYL TRANSFERASE II

Fraction	*Volume (ml)*	*Protein (mg)*	*Total activity (units)*		*Specific activity (units/mg)*		*Ratio Assay A/Assay B*
			Assay A	*Assay B*	*Assay A*	*Assay B*	
DEAE-Cellulose	23	89	2799	5.67	31.3	0.063	490
4-Aminobutyl-Sepharose IIa	6.2	7.7	1285	2.93	166.5	0.380	438
IIb	3.7	1.5	1170	2.66	780.0	1.77	439
Bio Gel $A_{1.5m}$ (IIa)	6.8	2.3	863	2.57	377.0	1.12	335

Fractions (33–55) containing branching activity were pooled and concentrated by ultrafiltration. The concentrated fraction was dialyzed overnight against 1 liter of buffer. This fraction was applied to a buffer-equilibrated column (2.5 × 33 cm) of Bio-Gel $A_{1.5m}$ and the enzyme eluted with buffer (the enzyme was eluted as a single symmetrical peak and fractions were pooled and concentrated as before). Table II summarizes the purification of branching-enzyme I after chromatography on DEAE-cellulose. At each step of purification, the ratio of activity (Assay A/Assay B) did not vary significantly (Table II).

Further purification of maize branching enzyme II. — DEAE-cellulose Fraction II was adsorbed onto a column (1.5 × 18 cm) of 4-aminobutyl-Sepharose equilibrated with 50mM Tris–acetate buffer containing 2.5mM 1,4-dithiothreitol, 10mM EDTA, and 5% sucrose. The column was washed with 175 ml of buffer and the enzyme eluted with an 800-ml, linear gradient of 0–1.0M potassium chloride in the same buffer (Figure 2B). Fractions 41–59 (Fraction IIa) and also fractions 60 to 80 (Fraction IIb) were pooled, concentrated, and dialyzed overnight against buffer. Fraction IIa was applied to a column (2.5 × 33 cm) of Bio-Gel $A_{1.5m}$ and eluted with buffer. A single, symmetrical peak was observed, and peak fractions were pooled and concentrated by ultrafiltration. A summary of the purification of branching-enzymes IIa and IIb

after chromatography on DEAE-cellulose is given in Table III. As with branching-enzyme I, subsequent purification did not significantly change the ratio (Assay A/Assay B) of activity for the two assay systems.

All branching-enzyme fractions were free of phosphorylase activity. Fractions IIa and IIb did contain low starch-synthase activities, 0.07 and 0.014% of the branching activity (Assay A), respectively. No amylolytic or R-enzyme activities could be detected in 2-h incubations with all fractions. However, a small release of reducing power in the R-enzyme assay was observed in 24-h incubations with branching enzymes IIa and IIb. All enzymes had broad pH optima (pH 6.0–8.0) when measured in citrate or phosphate buffers (0.1M) by Assay B. However, Fraction I was most active in phosphate buffer, whereas branching-enzymes IIa and IIb were most active in citrate buffer. All enzymes showed low activity (less than 15%) in 0.1M Tris–Cl^- buffer over the same pH range. Native molecular weights were estimated by gel filtration (Bio-Gel $A_{1.5m}$) to be 70,000–90,000 for branching-enzymes I and IIa.

Gel electrophoresis of branching enzymes. — The Bio-Gel $A_{1.5m}$ fraction of branching-enzyme I had three protein-staining bands after disc-gel electrophoresis. Protein was applied at 25 μg per gel. A single band of activity having an R_m (relative migration value to the tracking dye) of 0.096 corresponded to the major protein-band. No activity was observed with two faster moving, minor bands. Branching-enzymes IIa and IIb showed 2 protein-staining bands in disc-gel electrophoresis. Both bands corresponded to activity bands having R_m values of 0.42 for the major band and 0.56 for the minor band. No additional protein bands were observed. Electrophoresis of 20 μg of protein of the branching-enzyme fractions in dodecyl sodium sulfate in a discontinuous system showed a single protein-band for branching-enzyme IIb and a major protein with two slower migrating, minor bands for branching-enzyme IIa. A major protein-band with 2–4 minor bands was observed for branching enzyme I. The electrophoretic mobility of the major bands in dodecyl sodium sulfate gels were compared with standard proteins subjected to electrophoresis for the same time on separate gels. A plot of R_m versus the logarithm of the molecular weights of the standards (fumarase, bovine serum albumin, and phosphorylase *a*) was linear. The R_m value of 0.31 obtained for both branching-enzymes IIa and IIb corresponded to a molecular weight of 80,000. The R_m value of 0.26 found for branching-enzyme I corresponded to a molecular weight of 89,000.

Action of branching enzymes. — The branching action of the purified branching-enzymes was tested on amylose and amylopectin by monitoring the reactions by the decrease in absorbance and wavelength maxima of the glucan–iodine complexes. All three enzymes decreased the absorption and wavelength maxima of the amylose–iodine complex (Table IV). However, branching-enzyme I caused the greatest change in 2 h. All enzymes showed hyperbolic kinetics when the change in absorbance at 660 nm was measured with various concentrations of amylose. K_m values of 160 and 500 μg/ml were found for branching-enzymes I and II (a and b), respectively. Branching-enzymes I and IIb, but not IIa, caused decreases in the absorbance and wavelength maxima of the amylopectin–iodine complex in 2 h (Table IV). However, branching-

enzyme IIa was shown to be also active with amylopectin upon longer incubation, and decreases in the overall absorbance as well as wavelength maxima were observed.

Branched α-D-glucans were also formed by the combined action of the branching enzymes and rabbit-muscle phosphorylase *a* at enzyme ratios of 12:1. All three branching-enzymes stimulated the incorporation of D-glucose into the growing D-glucan (Fig. 3). In reactions containing branching-enzymes I and IIb, 60–70% of the total D-glucose was incorporated in 2 h. However, mixtures containing branching-enzyme IIa incorporated only 35% in the same time-period. In mixtures containing all three enzymes (equal activities), intermediate incorporation of D-glucose was observed (Fig. 3). The reactions were also monitored by measuring the absorption spectra (400–700 nm) of the iodine complexes of the growing α-D-glucans (Fig. 4). All reactions were characterized by the rapid formation (within 30 min) of an α-D-glucan product showing maximal absorbance. The absorbance and wavelength

TABLE IV

THE ACTION OF MAIZE BRANCHING-ENZYMES ON NATURAL α-D-GLUCANS OR SYNTHETIC α-D-GLUCAN FORMED BY THE COMBINED ACTION OF THE BRANCHING ENZYMES AND RABBIT-MUSCLE PHOSPHORYLASE *a*. TIME OF TREATMENT WAS 120 MIN

Substrate	*λ_{max} of iodine stain/% decrease in iodine stain at 660 nm*			
	Before treatment	*+ Fraction I*	*+ Fraction IIa*	*+ Fraction IIb*
Amylose	630	530/87.4	585/41.4	580/50
Amylopectin	535	505/63.9	535/0	510/68.7
+ phosphorylase *a*	—	485	485	460

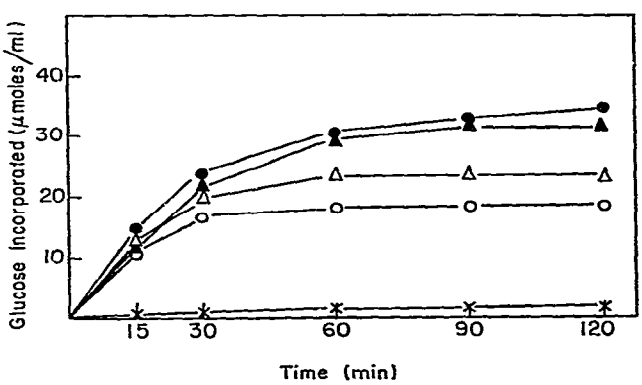

Fig. 3. Formation of D-[^{14}C]glucan catalyzed by the simultaneous action of maize branching-enzymes and phosphorylase *a* from α-D-[^{14}C]glucosyl phosphate, with a ratio of branching-enzyme activity to phosphorylase *a* activity of 12:1; (▲) branching-enzyme I, (○) branching-enzyme IIa, (●) branching-enzyme IIb, (△) equal activities of all three branching enzymes, (×) phosphorylase *a* control. The procedure is described in the text; (Assay B) and reaction volumes were scaled up to a total volume of 3.0 ml.

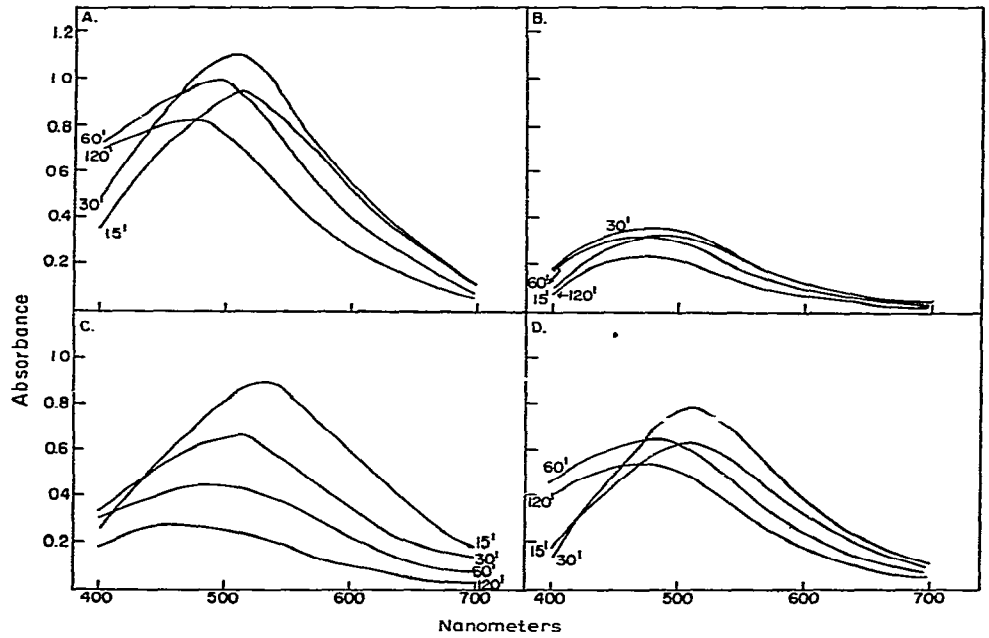

Fig. 4. Absorption spectra of α-D-glucan–iodine complexes of D-glucans formed by the simultaneous action of maize branching-enzymes and phosphorylase *a*, with an activity ratio of 12:1. A, branching-enzyme I; B, branching-enzyme IIa; C, Branching-enzyme IIb; D, equal activities of all three branching enzymes. The procedure is described in the Methods. No absorbance was observed at zero time.

maxima of the iodine complexes of these glucans then decreased as further branching occurred. This fall occurred during a period, 30–120 min, during which net synthesis had slowed (Fig. 3). Reactions containing branching-enzyme IIb differed from others by showing the greatest absorbance at 15 min, as compared to 30 min for other reactions. The wavelength maxima of the iodine complexes of the glucans at the time of greatest absorption ranged from 490–530 nm in all reactions. As further branching occurred, the wavelength maxima decreased to 450–485 nm as the overall absorbance dropped.

Glucan products were isolated after 120 min. These products were further characterized by debranching with *Cytophaga* isoamylase, by unit chain-length estimation, and by size estimation by gel filtration. Average unit chain-lengths of 24, 25, and 16 were found for glucans from reactions with branching-enzymes I, IIa, and IIb, respectively. All glucans as well as natural amylopectin were of high molecular weight (greater than 10^7), as shown by exclusion from Bio-Gel A_{50m} (Fig. 5). The product formed from the combined action of all three enzymes and phosphorylase was also excluded from Bio-gel A_{50m} (not shown). However the product formed by the branching of amylose by branching-enzyme I was included in gel filtration and, as expected, does not appear to be larger than the original substrate (Fig. 5).

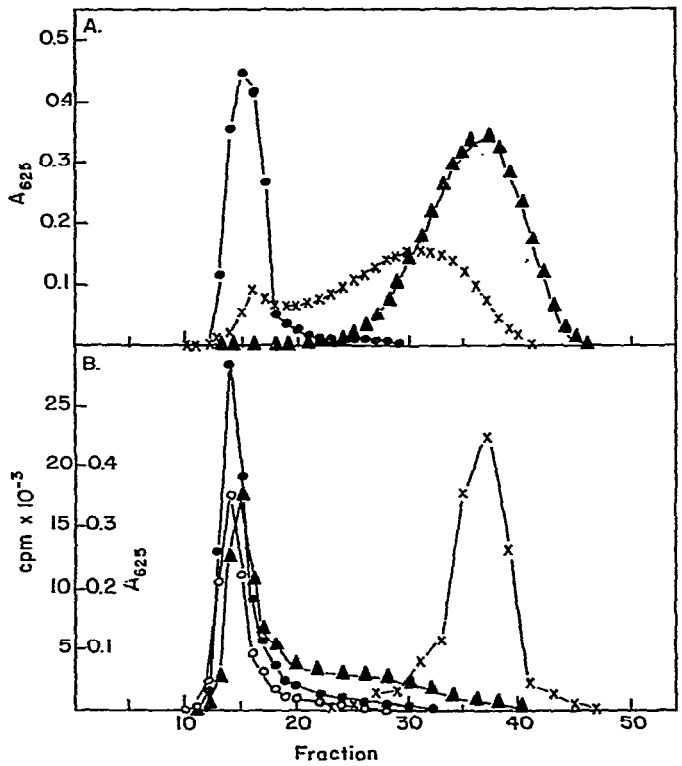

Fig. 5. Gel filtration of α-D-glucan products of maize branching-enzymes on Bio-Gel A_{50m}. A. Chromatography of standard glucans; (×) Blue Dextran, (●) amylopectin, (▲) amylose. B. Chromatography of α-D-glucan products; (▲) glucan formed by the combined action of branching-enzyme I and phosphorylase *a*, (○) glucan formed by the action of branching-enzyme IIa and phosphorylase *a*, (●) glucan formed by the combined action of branching-enzyme IIb and phosphorylase *a*, (×) amylose branched by branching-enzyme I. The procedure is described in the text.

DISCUSSION

The use of two distinct assay-systems for detection of branching enzymes has enabled us to identify multiple forms of branching enzyme in developing maize kernels. These forms do not appear to be tissue-specific; no differences were observed in enzyme preparations in which embryos and scuttelum were removed from the kernels as compared with preparations in which embryos and scuttelum had not been removed. Through the use of aminoalkyl-Sepharose columns, we have been able to purify the different forms to a high degree. DEAE-Cellulose fractions were purified twofold for branching-enzyme I and 5–20-fold for branching-enzyme II (a and b). Other α-D-glucan biosynthetic enzymes have been purified by using these resins as an absorbent[8,16,17], and the aminoalkyl-Sepharose resins appear to be of general utility in the purification of many of these enzymes.

Two major peaks of branching-enzyme activity were observed by chromatography on DEAE-cellulose (Fig. 1). Branching-enzyme I was not absorbed onto

DEAE-cellulose, but branching-enzyme II was absorbed and was coeluted with both the unprimed and primed starch-synthase activities. During further purification, branching-enzyme II was eluted from 4-aminobutyl-Sepharose as two peaks of activity (Fig. 2B). These two fractions, IIa and IIb, could not be distinguished in the two assay systems (Assays A and B), or by disc-gel electrophoresis with activity staining, or by dodecyl sodium sulfate electrophoresis. The only differences observed for the two fractions were, with fraction IIb, a more rapid branching of amylopectin (Table IV) and greater incorporation of D-glucose into D-glucan in simultaneous reactions with phosphorylase *a*. The significance or relationship of branching-enzymes IIa and IIb is not yet clear.

Major differences were observed between branching-enzyme I and branching-enzyme II (a and b collectively). Branching-enzyme I had the greatest activity when the enzymes were assayed by the branching of amylose (Assay B), but the situation was reversed when the enzymes were assayed by the stimulation of phosphorylase *a* (Assay B). Ratios of activity (Assay A/Assay B) 30–50 and 250–500 were found for branching-enzymes I and II, respectively, for various preparations of the fractions. Variation in these ratios resulted from high K_m values for amylose as a substrate (160 and 500 μg/ml, respectively, for branching-enzymes I and II), as well as the difficulties in handling amylose solutions of high concentration. Not only did branching-enzyme I not bind DEAE-cellulose, but it also failed to bind to 4-aminobutyl-Sepharose, whereas branching-enzyme II was absorbed onto both resins. Similarly, branching-enzyme I gave a very different pattern of activity-staining in disc-gel electrophoresis as compared to the pattern observed for branching-enzyme II. Both branching-enzyme I and II did, however, behave as monomers as determined by gel filtration and dodecyl sodium sulfate-gel electrophoresis, although branching-enzyme I was estimated to be 10% larger. Other branching enzymes, including those of potato[18] and *E. coli*[17], have been observed to be monomeric and have molecular weights around 85,000.

All three branching enzymes could branch amylose as well as add additional branch points to amylopectin. Similarly, purified, potato branching-enzyme exhibited a similar pattern of action[18]. In addition, all three branching enzymes were shown to interact with phosphorylase *a* to catalyze the formation of high molecular weight, highly branched D-glucans from α-D-glucosyl phosphate. Although average unit chain-lengths of the glucans, as determined by debranching with isoamylase, were near those of natural amylopectins (20–26), none of the glucans formed had spectral qualities (wavelength maxima) when complexed with iodine of amylopectin. It is not surprising that these preliminary experiments failed to produce a polysaccharide resembling amylopectin. Not only is phosphorylase a nonphysiological D-glucan synthase[9], but multiple forms of starch synthase (primed and unprimed) have been reported for numerous species[6,19,20]. Therefore, the biosynthesis of amylopectin may not depend on simply two enzymes (starch synthase and branching enzyme), but may involve several isoenzymes having distinct reaction mechanisms. As a result,

the final understanding of the biosynthesis of amylopectin may require the characterization of an interaction of a very complicated spectrum of enzymes.

Although multiple forms of branching enzymes have been shown by poly(acrylamide)-gel electrophoresis in a number of species[21], the isolation of multiple forms from nonmutant lines of maize has not been reported. Multiple forms of branching enzyme have been observed in spinach leaves[6]. As with the maize enzymes, one form was associated with starch-synthase activity and the other was not. However, the only difference reported between the two enzymes was relative activities in different buffers. This is the first report of the isolation of major, multiple forms of branching enzymes having different branching activities. Previous failure to detect the multiple forms of branching enzymes may be due, in part, to the insensitivity of the commonly used branching assay for the amylose–iodine complex. The significance of the different forms of branching enzyme in the biosynthesis of amylopectin must, however, await more thorough studies of branching mechanisms and studies of the interactions of these enzymes with the primed and unprimed starch synthases.

As a final note of proof of the differences of branching-enzymes I and II, preliminary studies of the two independent, nonlinked mutants of maize (*amylose-extender*; *ae* and *sugary*; *su*) have indicated that the two branching enzymes may be genetically independent as well. Chromatography on DEAE-cellulose of extracts of *ae* (which conditions the formation of starch having a less-highly branched amylopectin[22]) gives normal amounts of branching-enzyme I and decreased amounts of branching-enzyme II, with low activity only associated with the primed starch-synthase activity. Furthermore, it has been impossible to demonstrate differences between samples of branching-enzyme I from *ae* and nonmutant extracts. *Sugary* endosperm has long been known to contain a large proportion of the highly branched, water-soluble, starch-related material termed phytoglycogen[23]. Chromatography on DEAE-cellulose of extracts of *su* have shown similar patterns of branching enzymes as nonmutant. However, the activity of branching-enzyme I is enhanced as compared to nonmutant branching-enzyme I, and very different patterns of the branching of amylose have been observed. Thus branching-enzymes I and II appear to be under independent genetic control. Final elucidation of the genetic control of the branching enzymes, combined with more-thorough studies of branching mechanisms, as already mentioned, should be important in the final understanding of the biosynthesis of amylopectin.

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