Carbohydrate Research, 107 (1982) 215–230
Elsevier Scientific Publishing Company, Amsterdam –Printed in The Netherlands

BRANCHING ENZYME FROM AMYLOMAIZE ENDOSPERMS*

TADASHI BABA**, YUJI ARAI, TOMIO ONO, AKEMI MUNAKATA, HIROAKI YAMAGUCHI, AND TATSURO ITOH

Laboratory of Metabolic Chemistry, Institute of Applied Biochemistry, The University of Tsukuba, Ibaraki 305 (Japan)

(Received September 7th, 1981; accepted for publication, February 9th, 1982)

ABSTRACT

Branching enzyme [(1→4)-α-D-glucan:(1→4)-α-D-glucan 6-glucosyltransferase, EC 2.4.1.18] from developing amylomaize endosperms was fractionated by precipitation with ammonium sulfate and separated into two forms by chromatography on DEAE-cellulose. One form was purified to homogeneity, and found to be primarily monomeric with a broad pH-optimum (pH 6.5–8.0), a low activity in Tris-HCl buffer, and a molecular weight of ~90,000. These properties were similar to those of a branching enzyme from normal maize kernels previously described. The purified enzyme was apparently inhibited by a low concentration of mercury(II) chloride and increasing concentrations of citrate. The K_m value of the enzyme was 18 μg/mL for potato amylose. Further purification of another form of amylomaize branching-enzymes implied the possible presence of additional proteins having molecular weights of ~92,000 and ~45,000. The total content of branching enzyme in amylomaize endosperm was approximately one-third of that present in normal maize during endosperm development. The results suggest that amylomaize endosperms may contain at least three forms of branching enzyme, and that the decreased content of amylopectin fraction in amylomaize starch may be associated with the decrease in the total content of branching enzyme.

INTRODUCTION

The biosynthesis of the (1→4)-α- and (1→6)-α-D-glucosidic bonds of starch in plants is usually considered to be catalysed by two starch synthetases, namely, soluble starch-synthetase [ADP-D-glucose:(1→4)-α-D-glucan 4-α-D-glucosyltransferase, EC 2.4.1.21] and starch granule-bound starch-synthetase [nucleoside diphosphoglucose:(1→4)-α-D-glucan 4-α-D-glucosyltransferase], and by a plant branching-enzyme [(1→4)-α-D-glucan:(1→4)-α-D-glucan 6-glucosyltransferase, EC

*Role of the Recessive *Amylose-extender* Allele in Starch Biosynthesis of Maize, Part II. For Part I, see ref. 21.

**To whom correspondence should be addressed.

2.4.1.18], although participation of some other enzymes cannot be ruled out. The purification and some properties of the starch-synthesising enzymes have been investigated for maize (*Zea mays*) kernels[1-5], potato (*Solanum tuberosum*) tubers[6-8], rice (*Oryza sativa*) grains[9-11], and other plant tissues[12-15].

Storage starch of various plants, regardless of the species, normally contains two major components, namely, ~25% of amylose and ~75% of amylopectin. However, the relationship between these proportions and the properties of the starch-synthesising enzymes is not understood. For example, how does part of the amylose in the starch evade attack by the branching enzyme *in vivo*?

Among many endosperm mutants of maize, amylomaize is especially noted either for an increased content of amylose, or for an altered structure of amylopectin in the endosperm starch[16-20]. These facts seem to imply that the starch-synthesising enzymes are influenced qualitatively and/or quantitatively by the recessive *amylose-extender* (*ae*) allele during the biosynthesis of amylomaize starch. We reported[21] variations in the disc-gel electrophoretic patterns for multiple forms of soluble starch-synthetase due to the recessive *ae* allele. The slow-moving bands of the starch synthetase were observed only in the presence of the *ae* allele and at limited stages of endosperm development. In addition, a fast-moving starch synthetase synthesised a product whose iodine complex had a λ_{max} value that changed to longer wavelengths with increasing doses of *ae*. Boyer and Preiss[5] also reported that amylomaize kernels were deficient in one of the three forms of branching enzyme found in normal maize, and suggested that the absence of the branching enzyme might result in fewer branch points in amylomaize amylopectin. Thus, it seems possible that the recessive *ae* allele produces these qualitative effects on certain enzymes implicated in starch biosynthesis. However, quantitative changes of the starch-synthesising enzymes during amylomaize-starch biosynthesis, and detailed characterisations of branching enzymes from amylomaize endosperms, have not been reported.

We have now characterised the branching enzymes from developing amylomaize endosperms, and have compared the content of branching enzyme in normal and amylomaize endosperms during development. The overall purpose of this investigation was to elucidate, in further detail, the role of the recessive *ae* allele in the starch biosynthesis of maize.

EXPERIMENTAL

Materials. — The dent inbred M-14 homozygous seeds for *ae* and its non-mutant were obtained from Dr. E. Amano (National Institute of Genetics, Misima, Sizuoka, Japan), and they were field-grown in 1980 at The Tsukuba Agricultural Technical Center in our University. Developing kernels of each genotype were harvested at 10, 14, 18, 22, 28, and 36 days after self-pollination, quickly frozen on "Dry Ice", and then stored at −20° until used[22]. Potato amylose (Type III) and rabbit-muscle phosphorylase *a* (twice crystallised and lyophilised powder) were purchased from Sigma, $\overline{\text{d.p.}}$(average degree of polymerisation)-17 and $\overline{\text{d.p.}}$-100

amylose were obtained from Hayashibara (Okayama, Japan), and D-glucose 1-phosphate was obtained from Boehringer Mannheim. All other reagents were of the highest purities available.

Preparation of affinity resins. — An affinity resin with $\overline{d.p.}$-17 amylose attached to Sepharose 4B (Pharmacia) was prepared according to the method of Matheson and Richardson[23]. Washed Sepharose 4B (10 g) was activated by being stirred at room temperature in 0.2M Na_2CO_3 (40 mL) containing cyanogen bromide (1 g), and the pH of the solution was maintained at 11.0 by dropwise addition of M NaOH. The activated Sepharose was immediately washed with ice-cold water (400 mL) and 0.2M $NaHCO_3$ (400 mL), and then suspended in 0.2M $NaHCO_3$ (40 mL) containing 4 g of diaminohexane (pH 9.5). The slurry was gently stirred overnight at 4°, filtered, and washed with 0.2M $NaHCO_3$ and water (400 mL of each). A solution of $\overline{d.p.}$-17 amylose (400 mg) in 0.2M $NaHCO_3$ (40 mL) was mixed with 80% cyanogen bromide in 100 μL of acetonitrile for 90 s. The ω-aminohexyl-Sepharose was added to the mixture and stirred overnight at 4°. The gel was then filtered off, washed with 0.2M $NaHCO_3$ and water (400 mL of each), and finally stored in water containing 0.02% of NaN_3 at 0–4°.

ω-Aminobutyl-Sepharose 4B was prepared as described by Shaltiel and Er-El[24]. The gel was also stored in water containing 0.02% of NaN_3 at 0–4° until used.

Assays for branching-enzyme activity. — The assay procedures were essentially similar to those described by Boyer and Preiss[4], and by Brown and Brown[25]. Incubations were conducted at 30° in glass tubes.

Assay A. The basis of the assay was a stimulation by branching enzyme in the direction of synthesis of α-D-glucan from D-glucose 1-phosphate catalysed[25] by rabbit-muscle phosphorylase *a*. The reaction mixture consisted of 0.1M sodium citrate (pH 7.0), mM AMP, 50mM D-glucose 1-phosphate, 40 μg of rabbit-muscle phosphorylase *a*, and enzyme in a final volume of 0.2 mL. The mixture was incubated for 60 min, and the reaction was terminated by the addition of 5% aqueous trichloroacetic acid (0.8 mL). The precipitate formed was removed by centrifugation, and the inorganic phosphate content of the supernatant solution was measured by the method of Fiske and SubbaRow[26]. One unit of enzyme activity was defined as 1 μmol of inorganic phosphate liberated from D-glucose 1-phosphate per min under these conditions.

Assay B. Branching-enzyme activity was also assayed by the decrease in absorbance of an amylose–iodine complex[4]. The reaction mixture consisted of 0.1M sodium citrate (pH 7.0), 200 μg of potato amylose, and enzyme in a final volume of 0.2 mL. Each aliquot (50 μL) sampled at time intervals was added with water (0.35 mL) to iodine reagent (2.6 mL). Absorbance of the D-glucan–iodine complex was recorded on a Hitachi Model 100-50 spectrophotometer over the range of 400–700 nm. One unit of activity was defined[4] as a decrease in absorbance of 1.0 at 660 nm per min. The iodine reagent was prepared according to the method of Krisman[27] except that the iodine solution was diluted with water instead of saturated $CaCl_2$ solution.

Assays for other enzyme activity. — Phosphorylase (EC 2.4.1.1) activity was measured in the direction of production of iodine-staining D-glucan, using soluble

starch (0.2%) as a primer[23]. Activities of alpha-amylase (EC 3.2.1.1) and R-enzyme (EC 3.2.1.41) were measured by the method of Drummond *et al.*[7].

Purification of branching enzyme. — All purification procedures were carried out at 0–4°. Endosperm tissues were prepared by removing embryo and pericarp from kernels harvested at 22 days after pollination. The endosperms (200 g) were ground with 100 mL of 50mM Tris-HCl buffer (pH 7.5) containing 5mM ethylenediaminetetra-acetic acid (EDTA) and 5mM 1,4-dithiothreitol (DTT) in a Waring Blendor for a few minutes. The homogenate was filtered through two layers of cheesecloth, and the remaining tissues were further ground repeatedly with more (2 × 100 mL) of the extracting buffer, using a mortar and pestle. After a third grinding, each filtered extract was combined and centrifuged at 28,000*g* for 20 min.

Step 1. Solid $(NH_4)_2SO_4$ was added to the crude extract to 0.5 saturation. The mixture was stirred for 2 h and centrifuged at 10,000*g* for 20 min. The supernatant solution was discarded, and the precipitate was dissolved in a minimum volume of 50mM Tris-HCl buffer (pH 7.5) containing 5mM EDTA and mM DTT, and then dialysed overnight against 6 L of the same buffer.

Step 2. The dialysed enzyme fraction was applied to a column (8.2 × 20 cm) of DEAE-cellulose (Whatman DE-52) that had been equilibrated with the above buffer. The column was washed with the same buffer (300 mL), and then the enzyme was eluted with a linear gradient of 0→0.4M NaCl in the same buffer (500 mL in each reservoir). Each fraction (15 mL) was collected at a flow rate of 40 mL/h and subjected to Assays A and B. Two peaks of branching-enzyme activity were observed as previously described[5] (Fig. 1). Branching-enzyme fraction I (B.E. I), which was not adsorbed on the first DEAE-cellulose column, was concentrated in an Amicon ultrafiltration 402 cell equipped with a Diaflo UM-20 membrane, and then dialysed overnight against 2 L of the same buffer.

Step 3. Fraction B.E. I was adsorbed on a column (1.6 × 10 cm) of $\overline{\text{d.p.}}$-17 amylose–Sepharose 4B which had been washed with 500 mL of 50mM Tris-HCl buffer (pH 7.5) containing 5mM EDTA, mM DTT, and 0.5M NaCl, and then equilibrated with the same buffer free of 0.5M NaCl. The column was thoroughly washed with the equilibrating buffer, and the enzyme was eluted with the same buffer containing $\overline{\text{d.p.}}$-17 amylose (0.5%). The active fraction of B.E. I was concentrated by the ultrafiltration system, and dialysed overnight against 2 L of 50mM Tris-acetate buffer (pH 9.2) containing 5mM EDTA and mM DTT.

Step 4. The dialysate of the affinity-column fraction was adsorbed on a column of 1 mL of DEAE-cellulose in a 3-mL syringe previously equilibrated with the above Tris-acetate buffer. The column was washed with the same buffer (20 mL), and the enzyme was eluted with a linear gradient of 0→0.1M NaCl in the same buffer (50 mL in each reservoir) at a flow rate of 20 mL/h. Fractions (2 mL) were collected and assayed. The active fractions were combined, carefully adjusted to pH 7.5 with M acetic acid, and concentrated by a Mini-Module NM-3 ultrafiltration system (Asahi Kasei Co. Ltd., Japan). The concentrate was then dialysed overnight against 2 L of 50mM Tris-acetate buffer (pH 7.5) containing 5mM EDTA and mM DTT, and the

dialysed solution was used as the final preparation of B.E. I. Chromatography on the second DEAE-cellulose column was done to separate B.E. I from d.p.-17 amylose, which was not adsorbed onto the DEAE-cellulose column.

Disc-gel electrophoresis. — A 7.5% polyacrylamide gel with Tris-glycine buffer system[28,29] was used. In some experiments, potato amylose (0.3 mg/mL) was polymerised in running gel, for the purpose of detection of the band of branching-enzyme activity[4]. After electrophoresis at 2 mA per tube at 0–4°, the active band was detected by incubation of the gels in 0.1M sodium citrate buffer (pH 7.0) for 2 h at 30°, followed by staining with an iodine reagent containing 0.2% of I_2 and 2% of KI in 0.01M HCl. Protein bands were detected by staining with Coomassie Brilliant Blue (Sigma).

Estimations of molecular weight. — Estimation of the molecular weight of branching enzyme by gel filtration on Sephadex G-150 was performed with a column (1.6 × 75 cm) previously equilibrated with 50mM Tris-HCl buffer (pH 7.5) containing 5mM EDTA, mM DTT, and 0.5M NaCl. The series of standard proteins used were rabbit-muscle aldolase (mol. wt. 158,000), bovine serum albumin (68,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and cytochrome C (12,500). Sodium dodecyl sulfate (SDS) gel electrophoresis was performed on 7.5% polyacrylamide gels according to the method of Weber *et al.*[30]. The same standard proteins, except for aldolase, were used to estimate the molecular weight of the branching-enzyme subunit. The protein bands were detected with Coomassie Brilliant Blue as described above.

Protein determination. — The method of Lowry *et al.*[31] was used with bovine serum albumin as the standard.

RESULTS

Purification of branching-enzyme fraction I. — This fraction (B.E. I) from developing amylomaize endosperms, which was not adsorbed on the first DEAE-

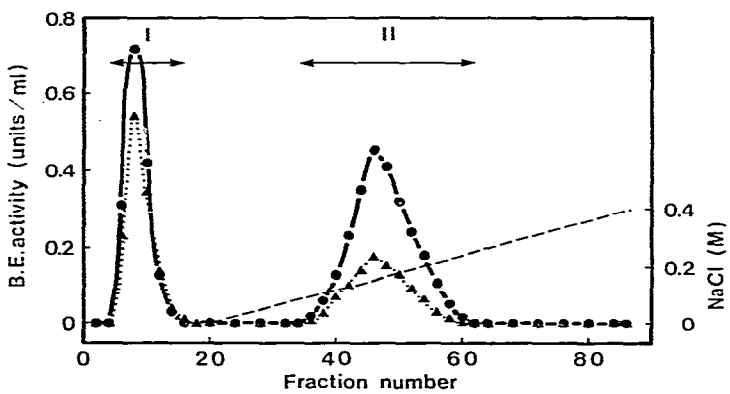

Fig. 1. Chromatography of amylomaize branching-enzymes on DEAE-cellulose: branching-enzyme activity, Assay A (—●—) and Assay B (..▲..); NaCl concentration (———). The procedure is described in the Experimental section.

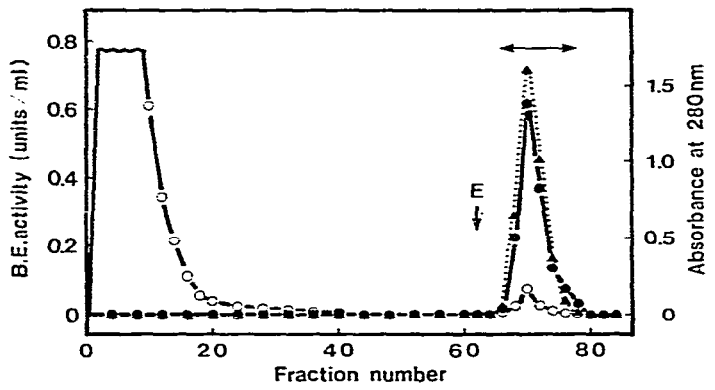

Fig. 2. Chromatography of B.E. I on d.p.-17 amylose–Sepharose 4B affinity column. The enzyme was eluted from the column with 0.5% of d.p.-17 amylose (E →). Fractions (9 mL) were collected at a flow rate of 40 mL/h and subjected to Assays A (—●—) and B (..▲..). Protein was followed by the absorbance at 280 nm (—○—).

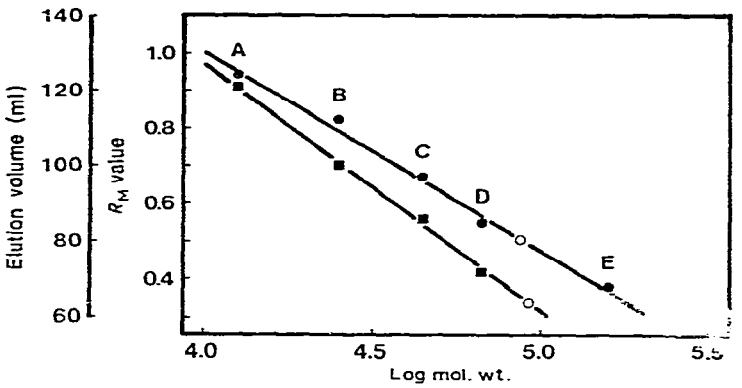

Fig. 3. Estimation of molecular weight of B.E. I by Sephadex G-150 gel filtration (—●—) and SDS gel electrophoresis (—■—). Standard proteins were A, cytochrome C; B, chymotrypsinogen A; C, ovalbumin; D, bovine serum albumin; E, rabbit-muscle aldolase. The values obtained for B.E. I are indicated by the open circles.

cellulose column (Fig. 1), was purified by an additional fractionation procedure including chromatography on an affinity column of d.p.-17 amylose–Sepharose 4B (Fig. 2) and a second column of DEAE-cellulose. Table I summarises the purification of amylomaize B.E. I. The final enzyme preparation was purified ~70-fold, with a 6.0% yield in Assays A and B, over the original $(NH_4)_2SO_4$ fraction. No activities of alpha-amylase and R-enzyme could be detected in the final preparation.

Properties of B.E. I. — (*a*) *Disc-gel electrophoresis.* Polyacrylamide disc-gel electrophoresis showed that, when ~30 μg of protein from the final B.E. I preparation was applied onto a 7.5% gel column (pH 8.9), only a single protein band having an R_m (relative migration value to the tracking dye) of 0.51 could be stained after electrophoresis under the conditions described in the Experimental section. When

TABLE I

PURIFICATION OF BRANCHING-ENZYME FRACTION I FROM AMYLOMAIZE ENDOSPERMS

Purification step	*Total protein (mg)*	*Total activity (units)*		*Specific activity (units/mg of protein)*		*Yield (%)*	
		Assay A	*Assay B*	*Assay A*	*Assay B*	*Assay A*	*Assay B*
1. $(NH_4)_2SO_4$	1210	67.3	60.5	0.06	0.05	100.0	100.0
2. First DEAE-cellulose	90.2	20.4	16.5	0.2	0.2	30.3	27.3
3. $\overline{D.p.}$-17 amylose–Sepharose	2.8	7.1	7.3	2.5	2.6	10.5	12.1
4. Second DEAE-cellulose	1.0	4.4	3.6	4.4	3.6	6.0	6.0

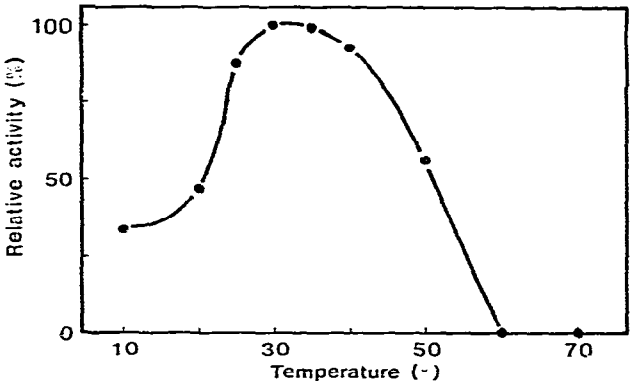

Fig. 4. Effect of temperature on B.E. I activity. The reaction mixture is described in the Experimental section as branching-enzyme Assay B. The enzyme concentration was 1.8 units/mL. Incubations were carried out for 60 min at the temperatures indicated.

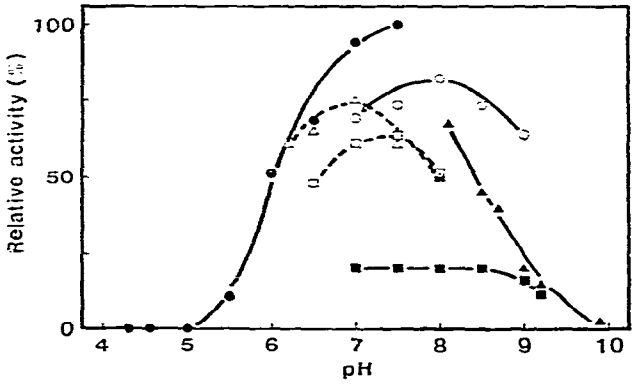

Fig. 5. Effect of pH on B.E. I activity. The enzyme solution (1.8 units/mL) was incubated at 30° for 60 min with potato amylose (1 mg/mL) in buffers at the pH values indicated. Sodium citrate (—●—), phosphate (--△--), Hepes–NaOH (--□--), Bicine–NaOH (—○—), Tris-HCl (—■—), and glycine–NaOH (—▲—) buffers were used at the concentration of 0.1M in the pH ranges 4.3–7.5, 6.2–8.0, 6.5–8.0, 7.0–9.0, 7.0–9.2, and 8.1–9.9, respectively. The enzyme activity was measured as branching-enzyme Assay B (see Experimental).

the running gel was polymerised in the presence of potato amylose, B.E. I had a strong affinity for the gel, and the electrophoretic mobility of the protein band was much lower (R_m 0.09) than that in the absence of the amylose. Although the running gel with amylose gave a bluish-purple stain with an iodine reagent, the enzyme-activity band could not be stained. This might be because the branching enzyme can act only in the direction of hydrolysis, on account of the fixation of amylose to the gel, and the severed fragments diffused outside the gel. However, the R_m value coincided with that of the protein band.

(*b*) *Molecular weight.* Duplicate estimates of the molecular weight of B.E. I, based on gel filtration on Sephadex G-150 and SDS-polyacrylamide gel electrophoresis, are shown in Fig. 3. The gel filtration gave a molecular weight of 85,000

±4,200. The SDS electrophoresis of the enzyme showed a single protein-staining band with a molecular weight of 92,000 ±4,200. Thus, the native B.E. I appeared to be a monomeric protein, as previously described[4,5].

(*c*) *Effect of temperature.* The temperature dependence of B.E. I activity in sodium citrate buffer at pH 7.0 is shown in Fig. 4. There is a temperature optimum of 25–40°, and ~35% of the maximum activity at 30° was observed even in an incubation at 10°. An incubation of the enzyme at 60° for 60 min caused a complete loss of the activity.

(*d*) *Effect of pH.* Fig. 5 shows that B.E. I has a broad pH-optimum that is dependent on the nature of the buffer used (0.1M each). A maximum activity was observed in sodium citrate, *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine), or phosphate buffers at pH 6.5–8.0. The activity in sodium citrate buffer at pH 6.0 was only 50% of that observed at the maximum at pH 7.5. Much lower activities were observed in Tris-HCl buffer.

(*e*) *Thermal stability.* As shown in Fig. 6, B.E. I was very sensitive to a temperature range between 50 and 60°. The enzyme was stable after heating at 50° and 53° for 30 min. Although ~50% of the original activity was retained after heating at 57° for 30 min, the enzyme was completely inactivated by pre-incubation at 58° for 5 min.

(*f*) *Effects of various metal ions and EDTA.* There was no significant inhibition and activation of B.E. I by Ca^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Li^{2+}, Mg^{2+}, and Zn^{2+} (at 5mM each) or by EDTA (up to 20mM). However, the enzyme activity was inhibited by a low concentration of $HgCl_2$ (not shown). The activity was decreased by ~50% at 60μM $HgCl_2$ and completely inactivated at 80μM.

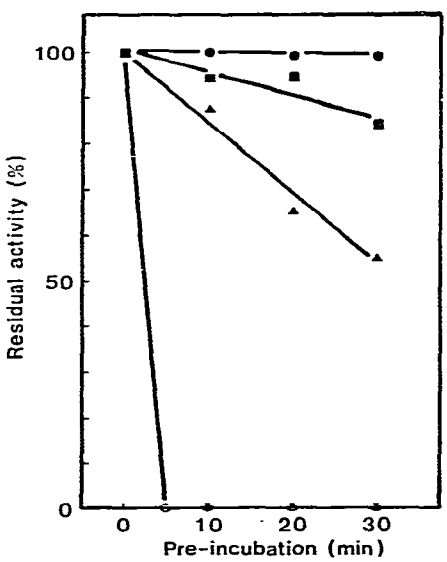

Fig. 6. Thermal stability of B.E. I. The enzyme solution (1.8 units/mL) was pre-incubated at 53° (—●—), 55° (—■—), 57° (—▲—), 58° (—○—), and 60° (—△—) for the times indicated. The residual enzyme activity was measured as branching-enzyme Assay B (see Experimental).

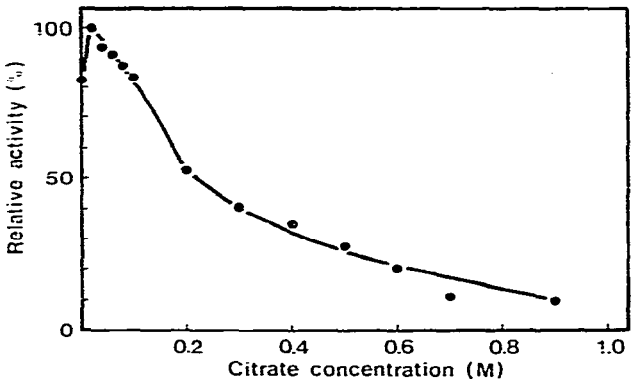

Fig. 7. Effect of citrate on B.E. I activity. The enzyme solution (1.8 units/mL) was incubated at 30° for 60 min in a mixture of 0.1M Hepes–NaOH buffer (pH 7.0) containing potato amylose (1 mg/mL) and various concentrations of citrate (0–0.9M). The residual activity was measured as branching-enzyme Assay B (see Experimental).

TABLE II

THE ACTION OF AMYLOMAIZE BRANCHING-ENZYME FRACTION I ON VARIOUS α-D-GLUCANS

Substrate	*λ_{max} of iodine stain (nm)*		*Decrease (%) in iodine stain at 660 nm*
	Before treatment	*After treatment*[a]	
Potato amylose	630	585	67
$\overline{\text{D.p.}}$-100 amylose	610	570	64
$\overline{\text{D.p.}}$-17 amylose	510	510	0
Waxy maize amylopectin	540	520	62
Soluble starch	565	545	67

[a]Reaction was carried out for 120 min at 30°, as branching-enzyme Assay B scaled up to a total volume of 1 mL. The enzyme concentration was 1.65 units/mL.

(*g*) *Effect of citrate.* Fig. 7 shows the effect of citrate at concentrations up to 0.9M on B.E. I activity in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)–NaOH buffer (pH 7.0). The maximum activity was observed at 20mM citrate, and the enzyme activity was inhibited by the increasing concentration of citrate. The activity was decreased by almost 10% at 0.9M citrate.

Action of B.E. I on various α-D-glucans. — As shown in Table II, the branching action of B.E. I on various α-D-glucans was tested by measurements of λ_{max} of the glucan–iodine stain before and after the enzyme treatment; each λ_{max} moved to a shorter wavelength, except for the $\overline{\text{d.p.}}$-17 amylose. For $\overline{\text{d.p.}}$-17 amylose, both the λ_{max} of the amylose–iodine complex and the absorbance of the iodine stain at 660 nm were not changed significantly by the B.E. I treatment. These results also indicated the absence of alpha-amylase from the final B.E. I preparation.

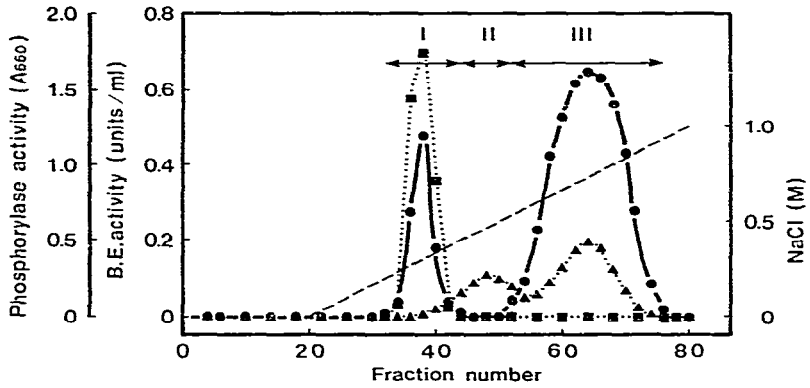

Fig. 8. Chromatography of B.E. II on ω-aminobutyl-Sepharose 4B. The enzyme was eluted with a linear NaCl gradient up to M (———). Each fraction (5 mL) was subjected to Assays A (—●—) and B (..▲..). Phosphorylase activity (..■..) was also assayed in each fraction from the column. The reaction mixture consisted of 0.1M sodium citrate buffer (pH 6.0), 0.1M D-glucose 1-phosphate, 0.2% of soluble starch, and enzyme in a final volume of 0.2 mL. The mixture was incubated for 10 min at 30°. An iodine reagent (3.8 mL) containing 0.01% of I_2 and 0.1% of KI in 0.2M acetate was added and the absorbance was measured at 660 nm.

The K_m value of B.E. I for potato amylose as a substrate was determined by the iodine-assay method under conditions where the assay had been shown to be linear with time for each concentration of the amylose used. The value from a plot of substrate concentration/initial velocity *vs* amylose concentration was 18 μg/mL, which was 10% of that reported previously[4] for normal maize B.E. I.

Further purification of branching-enzyme fraction II. — This fraction (B.E. II), which was adsorbed on the first DEAE-cellulose column and eluted with ~0.15M NaCl (Fig. 1), was concentrated by the Amicon ultrafiltration system, and then dialysed overnight against 4 L of 50mM Tris-HCl buffer (pH 7.5) containing 5mM EDTA and mM DTT. The dialysed enzyme solution was adsorbed on a column

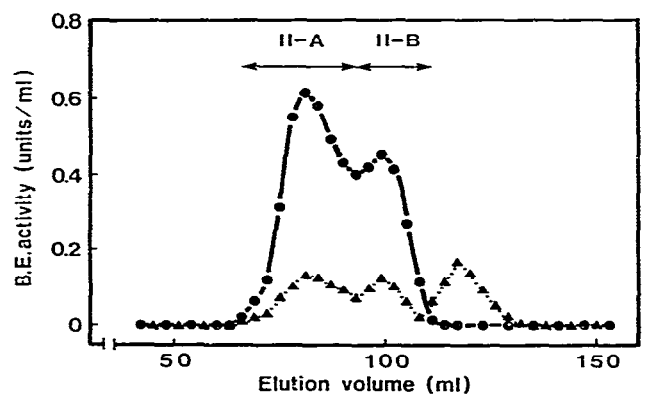

Fig. 9. Gel filtration of B.E. II on Sephadex G-150; branching-enzyme activity, Assay A (—●—) and Assay B (..▲..). The procedure is described in the Experimental section.

TABLE III

PURIFICATION OF BRANCHING-ENZYME FRACTION II FROM AMYLOMAIZE ENDOSPERMS

Purification step	*Total protein (mg)*	*Total activity (units)*		*Specific activity (units/mg of protein)*		*Yield (%)*	
		Assay A	*Assay B*	*Assay A*	*Assay B*	*Assay A*	*Assay B*
1. $(NH_4)_2SO_4$	1210	67.3	60.5	0.06	0.05	100.0	100.0
2. DEAE-cellulose	156.0	43.3	12.8	0.3	0.1	64.3	21.2
3. ω-Aminobutyl-Sepharose	5.3	25.5	5.4	4.8	1.0	37.9	8.9
4. Sephadex G-150 II-A	1.5	17.0	2.1	11.3	1.4	25.3	3.5
II-B	0.5	2.8	1.1	5.6	2.2	4.2	1.8

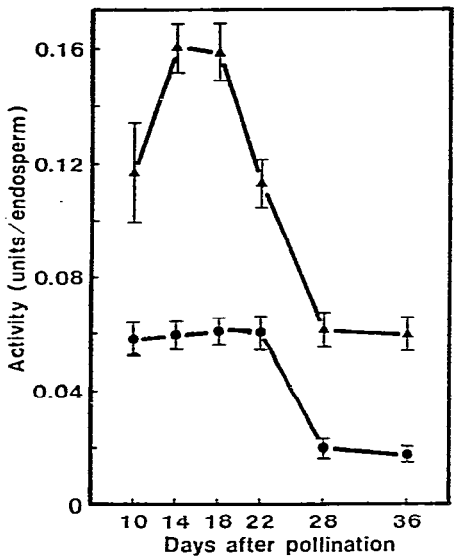

Fig. 10. Total content of branching enzyme during endosperm development. Endosperm tissues (10 g) were ground in a mortar with 10 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 5mM EDTA and mM DTT, and then filtered through two layers of cheesecloth. The homogenate was centrifuged at 43,000*g* for 20 min, and the precipitate was resuspended with 5 mL of the extracting buffer and then centrifuged again. The supernatant solutions were combined and dialysed overnight against the same buffer. The branching-enzyme activity in the dialysate from normal (—▲—) and amylomaize (—●—) endosperms was measured as Assay A (see Experimental). For each genotype and for each harvest date, at least five ears were used for the analysis of the enzyme content.

(1.4 × 13 cm) of ω-aminobutyl-Sepharose 4B that had been equilibrated with the same buffer. The column was washed with 100 mL of the equilibrating buffer, and the enzyme was then eluted with a linear gradient of 0→M NaCl in the same buffer (150 mL in each reservoir). Fractions (5 mL) were collected at a flow rate of 20 mL/h and subjected to Assays A and B. As shown in Fig. 8, two active peaks with Assay A, or Assay B, were observed in the elution profile. Two enzyme peaks (Peaks I and II) were eluted with 0.3 and 0.5M NaCl, and contained only activity detected by Assay A and B, respectively. A peak (Peak III) eluted with 0.7M NaCl showed both Assay A and B activities. In addition, phosphorylase activity was assayed in each fraction, and a single peak was observed, which coincided entirely with Peak I. The fractions of Peak III were combined, concentrated by the Mini-Module NM-3 ultrafiltration system, and then dialysed overnight against 2 L of 50mM Tris-HCl buffer (pH 7.5) containing 5mM EDTA, mM DTT, and 0.5M NaCl.

The dialysate of B.E. II was then filtered through a column (1.6 × 75 cm) of Sephadex G-150 that had been equilibrated with the same buffer. Fractions (2 mL) were collected at a flow rate of 10 mL/h and assayed. As shown in Fig. 9, the elution pattern indicated that B.E. II was separated into two branching-enzyme forms (B.E. II-A and II-B) differing in molecular weight. From a calibration using standard proteins on the same Sephadex G-150 column, the molecular weights of B.E. II-A

and II-B were estimated to be ~92,000 and ~45,000, respectively. Table III summarises the purification of amylomaize B.E. II.

Branching-enzyme content during endosperm development. — As shown in Fig. 10, whole branching-enzyme in endosperm was observed at all stages from 10 to 36 days after pollination. For both normal maize and amylomaize, the branching enzyme was present at the early stages rather than at the later stages of endosperm development. The enzyme content in amylomaize endosperm was approximately one-third of that in normal maize at certain stages of development.

DISCUSSION

Purified B.E. I from amylomaize endosperms had a molecular weight of ~90,000 (Fig. 3), a broad pH-optimum (pH 6.5–8.0), and a low activity in Tris-HCl buffer (Fig. 5). These properties are similar to those of the enzyme isolated [4] from normal maize kernels, and it is possible that B.E. I of amylomaize is identical with that of normal maize, as pointed out by Boyer and Preiss[5]. The amylomaize B.E. I was very sensitive to temperature (Fig. 6), and was not influenced by EDTA and various metal ions, except for Hg^{2+}. The inhibition of the enzyme activity at a low concentration of $HgCl_2$ showed a similarity to potato branching-enzyme reported by Drummond *et al.*[7].

Furthermore, the amylomaize B.E. I was apparently inhibited by increasing concentrations of citrate (Fig. 7). Of the multiple forms of soluble starch-synthetase from various plant sources, one form is characterised by its ability to synthesise a (1→4)-α-D-glucan in the absence of an added primer when a high concentration of citrate is present[2,3,6,14,15]. We also found that, with a form of soluble starch-synthetase specific for amylomaize endosperm, the enzyme activity could not be detected at low concentrations of citrate[21]. These findings may indicate that either stimulation or inhibition of the starch-synthesising enzymes by citrate should be regarded as having a possible physiological significance in starch biosynthesis, although there is no information about the relationship between the enzymes and citrate concentration *in vivo*.

The action of plant branching-enzymes has been reported[7,8,32,33]. Whelan[32] demonstrated that the minimum $\overline{\text{d.p.}}$ value of the linear chain on which potato branching-enzyme could act was ~40, and postulated that the branching enzyme required a double helix as a substrate for branching action and that the $\overline{\text{d.p.}}$ minima resulted from insufficient stability of the double helix when the chains had $\overline{\text{d.p.}}$ <40. In the work reported here, each λ_{max} of the iodine stains of various α-D-glucans was changed by treatment with amylomaize B.E. I, except for $\overline{\text{d.p.}}$-17 amylose (Table II). This result indicated that the minimum size of the linear substrate for the B.E. I was between $\overline{\text{d.p.}}$ 17 and 100, supporting the action of potato branching-enzyme[32].

Although amylomaize B.E. I did not act on $\overline{\text{d.p.}}$-17 amylose, the enzyme had an apparent affinity for the $\overline{\text{d.p.}}$-17 amylose when attached as a ligand to the Sepharose column (Fig. 2). Gibson *et al.*[34] found that unbranched oligosaccharides of the maltose

series with $\overline{d.p.} > 6$ inhibited branching-enzyme activity from rabbit skeletal muscle. It seemed likely that $\overline{d.p.}$-17 amylose might be an inhibitor for amylomaize B.E. I, and that the affinity between the enzyme and the amylose could be regarded as a mutual action due to the inhibitor. When rabbit-liver glycogen or soluble starch was attached to Sepharose, the enzyme was tightly adsorbed on the affinity column and could be eluted only with sodium chloride, resulting in ineffective purification.

The first chromatography on DEAE-cellulose showed the same elution order as that reported by Boyer and Preiss[5]. However, the elution profile of B.E. II from amylomaize endosperms on ω-aminobutyl-Sepharose was different from that reported[5], in that three enzyme peaks were detected using the dual assay system (Fig. 8). Of the three peaks, Peak I contained enzyme activity detected only by Assay A and which coincided with a peak of phosphorylase activity. An isozyme of maize phosphorylase that is capable of forming a polysaccharide in the absence of an added primer has been reported[35,36]. This finding, therefore, suggested that the conditions of Assay A were suitable for the phosphorylase which catalysed the unprimed reaction, and that the enzyme of Peak I, which was not revealed by Assay B, might correspond not to branching enzyme but to the phosphorylase. In fact, even when the Peak I enzyme was incubated under the conditions of Assay A free from rabbit-muscle phosphorylase *a*, approximately the same amount of inorganic phosphate as under the standard conditions could be detected in the reaction mixture.

Gel filtration of the Peak III enzyme, which was considered to correspond to branching enzyme, on Sephadex G-150 indicated that amylomaize B.E. II could be fractionated into two branching-enzyme forms, B.E. II-A and II-B, differing in molecular weight (Fig. 9). A recent report[4] indicated that B.E. IIa and IIb from normal maize kernels had molecular weights of 70,000–90,000. The molecular weight of amylomaize B.E. II-A agreed approximately with those of B.E. IIa and IIb from normal maize, and B.E. II-B had half the molecular weight of B.E. II-A. The final preparations of B.E. II-A and II-B were not homogeneous by disc-gel electrophoresis, and still contained alpha-amylase or R-enzyme activity. However, the present result seemed to imply that, like normal maize[4], amylomaize endosperms contain at least three forms of branching enzyme, namely, B.E. I, II-A, and II-B.

In comparison with normal maize, the total content of branching enzyme observed in amylomaize endosperm was much lower during its development (Fig. 10). The results of the present study suggest that the decreased content of the amylopectin component of amylomaize starch granules might be more closely related to the reduction of total content of branching enzyme rather than to qualitative changes of the enzyme. Furthermore, even if the structure of amylomaize amylopectin is characterised by fewer branch-points[5], the reduction of the content of branching enzyme in amylomaize also seems likely to lead to an altered structure. Additional, comparative investigations on the branching enzyme from normal and amylomaize endosperms, including their detailed mechanism of action, are necessary for a further understanding of the role of the recessive *ae* allele in starch biosynthesis.

ACKNOWLEDGMENT

The authors thank Dr. Etsuo Amano for kindly supplying maize seeds and for helpful discussions.

REFERENCES

1 T. AKATSUKA AND O. E. NELSON, *J. Biol. Chem.*, 241 (1966) 2280–2286.
2 J. L. OZBUN, J. S. HOWKER, AND J. PREISS, *Plant Physiol.*, 48 (1971) 765–769.
3 S. SCHIEFER, E. Y. C. LEE, AND W. J. WHELAN, *Carbohydr. Res.*, 61 (1978) 239–252.
4 C. D. BOYER AND J. PREISS, *Carbohydr. Res.*, 61 (1978) 321–334.
5 C. D. BOYER AND J. PREISS, *Biochem. Biophys. Res. Commun.*, 80 (1978) 169–175.
6 J. S. HAWKER, J. L. OZBUN, AND J. PREISS, *Phytochemistry*, 11 (1972) 1287–1293.
7 G. S. DRUMMOND, E. E. SMITH, AND W. J. WHELAN, *Eur. J. Biochem.*, 26 (1972) 168–176.
8 D. BOROVSKY, E. E. SMITH, AND W. J. WHELAN, *Eur. J. Biochem.*, 59 (1975) 615–625.
9 T. MURATA, T. SUGIYAMA, AND T. AKAZAWA, *Biochem. Biophys. Res. Commun.*, 18 (1965) 371–376.
10 Y. TANAKA AND T. AKAZAWA, *Plant Cell Physiol.*, 12 (1971) 493–505.
11 L. C. BAUN, E. P. PALMIANO, C. M. PEREZ, AND B. O. JULIANO, *Plant Physiol.*, 46 (1970) 429–434.
12 A. DOI, K. DOI, AND Z. NIKUNI, *Biochim. Biophys. Acta*, 113 (1966) 312–320.
13 A. DOI, *Biochim. Biophys. Acta*, 146 (1967) 603–605.
14 J. L. OZBUN, J. S. HAWKER, AND J. PREISS, *Biochem. J.*, 126 (1972) 953–963.
15 M. KREIS, *Planta*, 148 (1980) 412–416.
16 M. L. VINEYARD AND R. P. BEAR, *Maize Genet. Coop. News Letter*, 26 (1952) 5.
17 I. A. WOLFF, B. T. HOFREITER, P. R. WATSON, W. L. DEATHERAGE, AND M. M. MACMASTERS, *J. Am. Chem. Soc.*, 77 (1955) 1654–1659.
18 E. M. MONTGOMERY, K. R. SEXTON, R. J. DIMLER, AND F. R. SENTI, *Staerke*, 16 (1964) 345–351.
19 C. MERCIER, *Staerke*, 25 (1973) 78–83.
20 W. BANKS, C. T. GREENWOOD, AND D. D. MUIR, *Staerke*, 26 (1974) 289–328.
21 T. BABA, Y. ARAI, E. AMANO, AND T. ITOH, *Staerke*, 33 (1981) 79–83.
22 T. BABA, T. YAMAMOTO, Y. ARAI, M. YOKOTA, AND T. ITOH, *Phytochemistry*, 20 (1981) 1513–1517.
23 N. K. MATHESON AND R. H. RICHARDSON, *Phytochemistry*, 15 (1976) 887–892.
24 S. SHALTIEL AND Z. ER-EL, *Proc. Natl. Acad. Sci. U.S.A.*, 70 (1973) 778–781.
25 B. I. BROWN AND D. H. BROWN, *Methods Enzymol.*, 8 (1966) 395–403.
26 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375–400.
27 C. R. KRISMAN, *Anal. Biochem.*, 4 (1962) 17–23.
28 L. ORNSTEIN, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321–349.
29 B. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404–427.
30 K. WEBER, J. R. PRINGLE, AND M. OSBORN, *Methods Enzymol.*, 26 (1972) 3–27.
31 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
32 W. J. WHELAN, *J. Jpn. Soc. Starch Sci.*, 23 (1976) 101–123.
33 D. BOROVSKY, E. E. SMITH, W. J. WHELAN, D. FRENCH, AND S. KIKUMOTO, *Arch. Biochem. Biophys.*, 198 (1979) 627–631.
34 W. B. GIBSON, B. I. BROWN, AND D. H. BROWN, *Biochemistry*, 10 (1971) 4253–4262.
35 C. Y. TSAI AND O. E. NELSON, *Plant Physiol.*, 43 (1968) 103–112.
36 C. Y. TSAI AND O. E. NELSON, *Plant Physiol.*, 44 (1969) 159–167.