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MAIZE LEAF AND KERNEL STARCH SYNTHASES AND STARCH BRANCHING ENZYMES*

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Key Word Index—*Zea Mays*, Poaceae; starch synthesis, ADP-glucose, α-1,4-glucan-4-glucosyl-transferase, Q-enzyme, branching enzyme

Abstract—Soluble starch synthases and branching enzymes were partially purified from developing leaves and kernels of maize using DEAE-cellulose chromatography. One form of starch synthase and two forms of branching enzyme were detected in leaves as compared to two forms of starch synthase and three forms of branching enzyme isolated from the kernels. The starch synthase fraction from the leaves and the first starch synthase fraction from the kernels showed greater activity in reactions containing various glycogens as primers than in those containing amylopectin. In addition, both were capable of synthesizing a polyglucan in the absence of an added primer but in the presence of sodium citrate and bovine serum albumin (citrate-stimulated starch synthesis). The second starch synthase fraction from kernels showed greater activity with amylopectin as primer and had no citrate-stimulated activity. We suggest that the leaf enzyme and endosperm starch synthase I are the same enzyme and that it is 'constitutively' expressed. Branching enzymes from leaves and kernels differed not only in their elution profiles but also their stimulation of phosphorylase *a* (assay A) and amylose branching (assay B) activities. A minor branching enzyme fraction from leaves (leaf branching enzyme I) eluted from the DEAE-cellulose column after the addition of a salt gradient, whereas branching enzyme I from kernels eluted in the buffer wash prior to the application of the gradient. However, the ratios of assay A to assay B suggested that branching enzyme I from leaves was catalytically similar to branching enzyme I from the kernels. The major leaf branching enzyme (branching enzyme II) eluted at the same position from the DEAE-cellulose column as endosperm branching enzyme IIa. These enzymes had similar ratios of activity (Assay A/Assay B). The cross reaction of leaf branching enzymes with antisera prepared against maize endosperm branching enzymes in immunodiffusion experiments and enzyme activity neutralization experiments further demonstrated the ralationship of the leaf and endosperm branching enzymes.

INTRODUCTION

Starch is the most abundant storage glucan in the plant Kingdom. Starch accumulates in the leaves of most green plants during the light period before being hydrolysed for transport as sucrose to reserved tissue during dark periods [1, 2]. Starch also comprises the primary component of the endosperm of the major cereal crops. The starch biosynthetic process in both photosynthetic and nonphotosynthetic tissues is believed to be catalysed by two enzymes. starch synthases and branching enzymes. Starch synthases (ADP-glucose: α-1,4-glucan-4-glucosyltransferase, E.C. 2.4.1.21) catalyse the formation of the α-(1,4)-glucosidic linkage. In this reaction, the D-glucosyl unit from ADP-glucose is transfered to the four position of a D-glucosyl unit of acceptor α-glucan molecules. The net result is the formation of a new α-(1,4)-bond in the acceptor compound, thus increasing the size of the acceptor by one glucose molecule [3]. Starch branching enzymes (α-1,4-glucan: α-1,4-glucan-6-glucosyltransferase, E.C. 2.4.1.18) catalyse the synthesis of α-(1,6)-linkages. The reaction proceeds by the hydrolysis of a α-(1,4)-bond, followed by the subsequent reattachment of the severed chain fragment to a primary hydroxyl group of the remaining or another α-(1,4)-glucan chain by a α-(1,6)-linkage [4]. The combined action of starch synthases and starch branching enzymes determine the structure of the starch molecules.

Multiple forms of soluble starch synthase and branching enzyme have been identified and characterized in the seeds [5–8] and leaves [9, 10] of several higher plant species. In general, one form of soluble starch synthase is capable of synthesizing a polyglucan without the addition of a glucan primer to the reaction mixture. Presently, structural, biochemical, and genetic investigations of starch granule formation are being performed in several laboratories to elucidate the mechanism of action of the multiple forms of these enzymes. Though progress has been made, direct evidence linking a particular enzyme fraction to a specific function in starch granule biogenesis remains elusive. It has been suggested that multiple forms of starch synthase and branching enzyme may interact to form enzyme complexes, which in turn have different specificities for elongation and branching [11].

Little attention has been given to the properties of starch synthases and starch branching enzymes in different tissues from a single species. Hawker and Downton

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[10] reported differences in the number of multiple forms of starch synthase and starch branching enzymes of maize leaves and seeds However, no properties for the enzyme fractions were reported. In this paper, the properties of soluble starch synthases and branching enzymes from leaves and kernels of the maize inbred line W64A are compared Comparisons are based on chromatographic, kinetic and immunological properties of the enzymes A preliminary report of this work has been presented [12]

RESULTS

Five days after emergence, soluble starch synthase and branching enzyme activities were detected in crude extracts from leaves of maize (Fig. 1) Activities were measured on a fresh weight basis from five to 20 days after emergence Primed starch synthase activity was constant from five to 20 days after emergence. The citrate-stimulated starch synthase activity continued to increase from five to 15 days after emergence before levelling off The amount of citrate-stimulated activity was several-fold

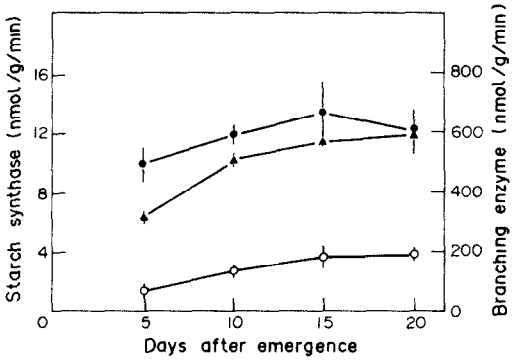

Fig 1 Starch synthase and branching enzyme activities from maize leaves at different times after emergence ●—●, primed starch synthase activity, ○--○, citrate-stimulated starch synthase activity, and ▲—▲, branching enzyme activity Vertical lines show the standard errors of the means

lower than the primed synthase activities. The activity of branching enzyme increased from five to 10 days, after which the activity remained constant (Fig 1)

Enzymes were purified from 20-day-old leaves in three experiments A typical purification of soluble starch synthase and branching enzyme is summarized in Table 1 Isolation was begun by homogenizing 100 g fresh weight of 20-day old maize leaves Similar results were obtained with 10 and 15-day old leaves (results not shown) Recoveries of primed starch synthase and branching enzyme activities from crude extracts of leaves after centrifugation were more than 80% and 85%, respectively (Table 1). Enzymes were further purified by ammonium sulphate at 40% saturation which complexed virtually all starch synthase and branching enzyme activity The extent of enzyme recovery was not altered when either PVP or Tris–HCl buffer, pH 8.5, was added to the extraction buffer Recoveries of branching enzymes and citrate-stimulated starch synthase activity greatly increased after DEAE-cellulose chromatography, probably indicating the elimination of contaminating amylase activity(s) which would interfere in branching enzyme and citrate-stimulated starch synthase assays Soluble starch synthases and starch branching enzymes from 22-day-old kernels were purified as above (results not shown) and the results were similar to those previously reported in detail [13]

DEAE-cellulose chromatography of the dialysed ammonium sulphate fractions from leaves and kernels is compared in Fig. 2 The elution profiles revealed distinct patterns for each tissue, and DEAE-cellulose fractions from leaves were numbered in the order of elution DEAE-fractions from kernels were pooled and labelled as described [13]. In leaf extracts, a single large peak of starch synthase (SS) eluted in the gradient at concentrations of 0 10–0 15 M KCl (Fig 2A), the same gradient concentration of KCl at which starch synthase I (SSI) from kernels eluted (Fig 2B) The kernel starch synthase II (SSII) eluted from the DEAE-cellulose column at 0 15–0 20 M KCl. Branching enzymes I and II from leaves eluted at 0.05–0 1 M KCl and 0 15–0 25 M KCl, respectively (Fig. 2A) Branching enzyme II eluted from the column at the same gradient concentration as that of

Table 1 Purification of branching enzymes and starch synthases from maize leaves

Fraction	Volume (ml)	Protein (mg)	Total activity (units*) BE†	Pr†	Cit-sti†	Specific activity (units/mg protein) BE†	Pr†	Cit-sti†
Crude	53 0	690	33	8 8	2 0	0 05	0 01	0 003
10000 *g*	47 5	300	29	7 1	0 1	0 10	0 02	0 001
40% $(NH_4)_2SO_4$ (Supernatant)	12 5	260	27	6 8	0 2	0 10	0 03	0 001
DEAE-cellulose								
I (37–51)	9 5	5 5	26	0 04	0 01	4 8	0 01	0 003
II (52–70)	13.5	75	0	2 7	4 9	.0	0 04	0 07
III (71–97)	18 0	59	150	1 5	0 9	2 5	0 03	0 02

*One unit of starch synthase activity is defined as nkat in the primed conditions (5 mg/ml amylopectin) or citrate-stimulated reaction conditions One unit of branching enzyme is expressed in nkat in the phosphorylase *a* stimulation assay (Assay A)

†BE, branching enzyme, Pr, primed starch synthase, Cit-sti, citrate-stimulated starch synthase

Numbers in parentheses are the fractions from DEAE-cellulose columns pooled for each fraction

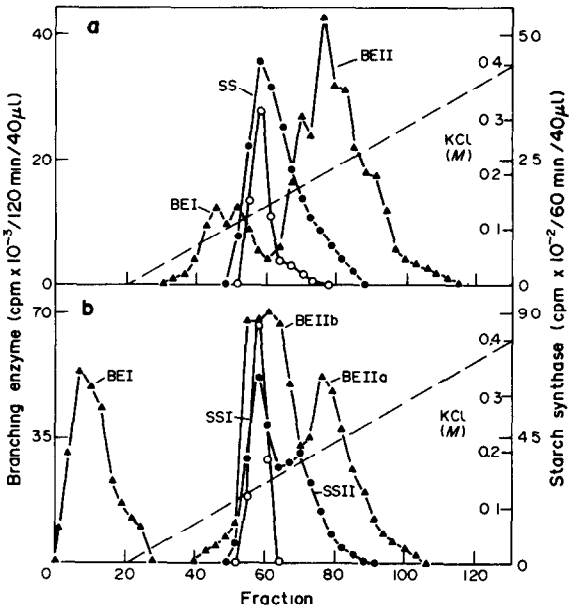

Fig 2. DEAE-cellulose chromatography elution profiles of starch synthases and branching enzymes a Leaf enzymes b Kernel enzymes ●—●, Primed starch synthase activity, ○—○, citrate-stimulated starch synthase activity, ▲—▲, branching enzyme activity (Assay A). The dashed line shows the concentrations of KCl at each position of the 0–0.4 M KCl gradient

kernel branching enzyme IIa (Fig. 2B). Leaf profiles differed from kernel profiles by the absence of a branching enzyme eluting before the KCl gradient (BEI) and at a KCl concentration in the gradient which elutes kernel BEIIb, and the absence of starch synthase II. In addition, kernels had no branching enzyme which eluted at the same gradient position as leaf branching enzyme I. Therefore, kernel BEI, BEIIb and SSII are not found in leaf extracts

Leaf starch synthase and kernel starch synthase I both catalysed citrate-stimulated starch synthesis (Fig. 2). In primed assays, leaf starch synthase and kernel starch synthase I showed greater activity with glycogen primers than amylopectin (Table 2). By contrast, kernel starch synthase II had no citrate-stimulated activity (Fig. 2) and greater primed activity with amylopectin as primer than glycogen (Table 2). The result for the kernel enzymes are similar to those previously reported [4, 5, 13, 14].

Table 2 Activity of maize leaf and kernel starch synthases with different glucan primers*

Enzyme source	Enzyme fraction	Activity (%)			
		AP†	RLG†	OG†	BLG†
Leaves	SS	100	563	183	144
Kernel	SSI	100	380	149	120
	SSII	100	86	62	35

*All primers were added at 5 mg/ml final concentration The activity of amylopectin was arbitrarily set at 100%.

†AP, amylopectin; RLG, rabbit liver glycogen, OG, oyster glycogen, BLG, beef liver glycogen

All multiple forms of branching enzymes isolated from leaves and kernels showed the ability to form α-(1,6)-linkages in amylose as measured by the altered absorbance patterns of the iodine-glucan complex (Table 3). The fall in absorbance at 660 mm was linear up to a 40% change during which enzyme activity was measured. During the assays, maximum absorbances for the iodine-glucan complexes fell to 540–590 nm, similar to the absorbance of amylopectin-iodine complexes. The activities of branching enzymes as measured by phosphorylase *a* stimulation (Assay A) and by amylose branching (Assay B) indicated ratios that were nearly equal not only between the branching enzyme I from leaves and kernels but also between leaf branching enzyme II and kernel branching enzymes IIa and IIb (Table 3).

When antisera prepared against maize endosperm branching enzymes [15] were used to neutralize leaf and kernel branching enzyme activities, similar neutralization patterns resulted (data not shown). Kernel branching enzyme I antiserum effectively cross reacted with only

Table 3 Summary of the properties of maize leaf and kernel branching enzymes

Enzyme source	Assay A*		Antiserum I		Antiserum IIa		Antiserum IIb	
	Assay B	λ_{max}†	Ouch.	Neutr	Ouch	Neutr.	Ouch.	Neutr.
Leaves								
BE I	92	590	+	43	–	ND⁺	–	ND
BE II	221	580	–	ND	+	66	+	12
Kernel								
BE I	99	540	+	97	–	ND	–	ND
BE IIa	236	580	–	ND	+	63	+	ND
BE IIb	208	580	–	ND	+	ND	+	6

*Ratio of branching enzyme activity as measured in Assay A and Assay B

†The maximum absorbance of the amylose-iodine complex after 2 hr of treatment with branching enzyme as assayed in Assay B.

Ouch., Ouchterlony double diffusion, Neutr, μl of antiserum required for neutralization of 50% the enzyme activity, –, no reaction detected, ND, not determined.

leaf branching enzyme I and kernel branching enzyme I (Table 3) For 50% neutralization of enzyme activity, 43 and 96 μl of antiserum per unit of enzyme activity was required for leaf and endosperm enzymes, respectively (Table 3) Antisera prepared against kernel branching enzymes IIa and IIb both cross reacted to leaf branching enzyme II with 66 μl and 12μl of antisera per enzyme unit required for 50% neutralization of enzyme activity, respectively (Table 3) No immunoprecipitate was observed when leaf branching enzyme I was tested against branching enzyme IIa and IIb antisera. Similarly, no immunoprecipitate was observed between leaf branching enzyme II and branching enzyme I antiserum

DISCUSSION

Multiple forms of starch synthase and branching enzyme have been reported in a variety of plant species [5–10]. To characterize further the properties of these enzymes and their tissue specificity, we have isolated these enzymes from maize leaves 20 days after emergence and from 22-day-old kernels of the maize inbred W64A The elution pattern of leaf enzymes from DEAE-cellulose chromatography (Fig. 2A) showed only one major starch synthase peak as opposed to two peaks as reported by Hawker and Downton [10]. Low starch synthase activity in maize leaves and a high phenolic content cannot be the cause of the absence of the second peak because high activity of starch synthase in maize leaves was reported in several studies [10, 16]. In addition, the addition of insoluble PVP to the purification buffers failed to reveal the second synthase peak No further separation was observed when the DEAE-fraction containing starch synthase from leaves was chromatographed on a diaminobutyl-Sepharose column (data not shown) Similarly, we observed two branching enzyme peaks on DEAE-chromatography and Hawker and Downton [10] observed three peaks. These differences are not easily resolved The only major differences are the varieties used in the two studies and the additional use of Assay A to measure branching enzyme in this study. Clearly, additional investigation into varietal differences is needed

The leaf starch synthase is similar to the kernel starch synthase I in several respects Both eluted from the DEAE-cellulose columns at similar positions in the gradient (Fig 2) Similarly both enzymes bound to diaminobutyl-Sepharose (data not shown). Both enzymes were capable of citrate-stimulated starch synthesis and were more active with glycogen primers (Table 2) Based on the evidence, we suggest that the leaf starch synthase and the endosperm starch synthase I are the same enzyme Ultimate proof of this suggestion will require comparison of leaf and endosperm enzymes at the molecular level The presence of the same enzyme in two distinct tissues leads us to suggest that this enzyme is 'constitutively' expressed in tissues active in starch synthesis The lack of a starch synthase II in the leaves may indicate an endosperm specific location of this enzyme. The examination of starch synthases in additional tissues will be necessary to verify these conclusions

In the characterization of branching enzymes, the ratios of branching enzyme activities as measured by Assay A and Assay B (Table 3) showed similarities not only between leaf branching enzyme I and kernel branching enzyme I but also between leaf branching enzyme II and endosperm branching enzymes IIa and IIb These relationships were further confirmed with Ouchterlony double diffusion and enzyme neutralization experiments The positions of leaf and endosperm branching enzymes I on DEAE-cellulose column profiles were the only differences observed for these enzymes Thus, these enzymes are related but not identical Based on the position of leaf branching enzyme II in DEAE-cellulose chromatography, we conclude that the leaf enzyme is identical to kernel branching enzyme IIa in respect to all properties examined If leaf BEII and endosperm BEIIa the same enzyme, this enzyme may be 'constitutively' expressed By contrast, we suggest that endosperm branching enzyme IIb may be endosperm specific

The observation of differing soluble starch synthases and branching enzymes in leaves and endosperm of maize is not surprising Starch synthesis in kernels is for storage over long periods of time By contrast, starch in leaves is transitory and is accumulated or degraded by dynamic regulated pathways. Although starch synthesis and branching enzymes are not allosterically regulated or rate-limiting in starch synthesis, these enzymes ultimately determine the structure of amylose, amylopectin and the starch granules Interestingly, genetic studies have often isolated mutants which appear to affect starch synthases only in maize endosperm (and pollen) but not leaves A possible explanation of these endosperm 'specific' mutant is the observation of endosperm specific isozymes reported here Work is in progress to determine the relationship of endosperm specific isozymes of starch synthase and branching enzymes and endosperm 'specific' mutants of maize

EXPERIMENTAL

Material Maize plants (*Zea mays* L) were grown in the greenhouse and leaves were harvested at 5, 10, 15 and 20 days after emergence and used immediately Plants were also grown in the field and ears were harvested at 22 days after pollination, quick frozen and stored at -20° until used In previous studies [13, 14], we have examined enzymes from field- and greenhouse-grown ears and found no differences [U-^{14}C] Glucose-1-phosphate and ADP-[U-^{14}C] glucose were obtained from Amersham/Searle Potato amylopectin was obtained from U S Biochemical Co Potato amylose, crystalline rabbit muscle phosphorylase a, rabbit liver glycogen Type III, and oyster glycogen were obtained from Sigma Maize endosperm branching enzyme antisera were produced as previously described [15]

Developmental study Washed fresh leaves (3–4 g) were ground in a pre-chilled mortar and pestle in cold 100 mM citrate buffer, pH 7 0, containing 5 mM dithioerythritol (DTE) (1 ml/g of tissue) and kept on ice Samples of the crude extract were used immediately to assay primed starch synthase activity (rabbit liver glycogen), citrate-stimulated starch synthase, and branching enzyme activity Enzyme activities are reported on a per g fr wt basis and are the means of four independent experiments with plants grown at different times

Enzyme assays Primed and citrate-stimulated starch synthase activities were measured by the incorporation of [^{14}C]glucose from ADP-[^{14}C]glucose into MeOH insoluble α-glucan in primed and unprimed (citrate-stimulated) reactions as described in ref [9] Branching enzyme activity was assayed based on the stimulation of α-D-glucan formation from [^{14}C]glucose-1-phosphate catalyzed by crystalline rabbit muscle phosphorylase *a* (Assay A) as described in ref [9] All assays performed at two enzyme concentrations in the range of linearity and included appropriate no enzyme and heat denatured enzyme controls

Amylose branching activity (Assay B) was measured by the decrease in absorbance at 660 nm of the amylose-I_2 complex [13]

Enzyme purification All procedures were performed at 0–4°. Following homogenization, crude extracts were filtered through two-layers of Miracloth and purified by centrifugation (30 min at 10000 *g*), $(NH_4)_2SO_4$ pptn (0–40% saturation of the supernatant), and DEAE-cellulose chromatography. A thorough description of buffers and purification procedures was described previously [13] In some experiments, the homogenization buffer was modified to contain 1% polyvinylpyrrolidone (PVP)

Protein was determined by the Lowry method [17] using BSA as standard.

Ouchterlony double diffusion plates. Specificity of the antisera was tested in several combinations with the leaf branching enzymes on double diffusion plates [18] The gels contained 1 mM K-Pi buffer (pH 7 0), 0 85% NaCl, and 1% Ionagar no. 2

Neutralization of branching enzyme activity with antiserum. All steps of the neutralization reaction were as described previously [15].

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