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STARCH SYNTHASE AND STARCH BRANCHING ENZYME FROM GERMINATING CASTOR BEAN ENDOSPERM

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Key Word Index—*Ricinus communis*; Euphorbiaceae; castor bean; starch biosynthesis; starch synthase; branching enzyme; starch phosphorylase; starch granule–bound starch synthase.

Abstract—Sucrose, but not starch, accumulates to high levels during fat utilization in young castor bean seedlings. High activities of starch synthase, starch branching enzyme and starch phosphorylase were present in the tissue but the very low ADPG pyrophosphorylase activity probably accounts for the low level of starch accumulation. DEAE-cellulose chromatography resolved a single ADPG starch synthase with both primed and citrate-stimulated (unprimed) activities. Three isozymes of branching enzyme were resolved, one co-eluting with starch synthase. ADPG and UDPG starch synthase activities were present in plastid-rich pellets (marker, RuBisCO). Treatment with {3-[(3-cholamido-propyl) dimethylammonio] 1-propane-sulphonate} (CHAPS) solubilized 75% of these activities. The remainder is attributed to starch granule-bound starch synthase. The effect of various glucan primers on the activities of soluble ADPG starch synthase and starch phosphorylase were determined.

INTRODUCTION

Reibach and Benedict [1] showed that five days after imbibition the endosperm tissue of castor bean seedlings contained > 100 mg free sugars, but only 1.1 mg starch per seedling. They showed that adenosine diphosphate glucose (ADPG) starch synthase was abundant in the tissue, while the activity of ADPG pyrophosphorylase was very low, and likely to limit starch accumulation.

Soluble starch synthase and starch branching enzyme have been found in multiple forms in seeds of starch accumulating plants, including maize [2, 3], teosinte [4], sorghum [5], rice [6], and *Pisum sativum* [7], potato tubers [8], and the leaves of maize [9] and spinach [10, 11]. Other enzymes capable of participating in starch synthesis include the starch granule-bound forms of starch synthase and starch phosphorylase.

In higher plants all enzymes involved in starch synthesis are confined to plastids [12]. The current investigation examined the nature of the starch synthases and branching enzymes in five-day-old castor bean endosperm for comparison with cereals and legumes which accumulate high levels of starch during development. The ability of soluble starch synthase and starch phosphorylase to utilize various primers was also studied.

RESULTS

The distribution of ribulose bisphosphate carboxylase (RuBisCO), primed ADPG starch synthase, and primed starch phosphorylase following differential centrifugation of a homogenate produced from five-day-old castor bean seedling endosperm is shown in Fig. 1. The bulk of the plastid marker enzyme, RuBisCO, was located in the 500 *g* and 15 000 *g* pellets. The proportion of unbroken cells in the 500 *g* pellet was calculated from the activity of the cytosolic marker enzyme, alcohol dehydrogenase. The levels of activity of RuBisCO, starch synthase, and phosphorylase were corrected for the presence of unbroken cells (Fig. 1). Most of the fumarase activity, a mitochondrial marker enzyme, was located in the 15 000 *g* pellet. Only 6.2% of the starch synthase activity and 16.5% of the phosphorylase activity were found in the 15 000 *g* supernatant, indicating that these starch-metabolizing enzymes are associated with organelles (Fig. 1).

Most of the RuBisCO activity observed in sucrose density gradients was associated with the pellets. ADPG pyrophosphorylase activity was exceedingly low in all sucrose gradient fractions assayed (maximum activity, 0.4 nmol/10 min/50 μl extract).

A separate preparation was used to isolate and partially purify the starch synthases and branching enzymes from young castor bean endosperm. Following anion-exchange chromatography of a dialysed and concentrated 40% ammonium sulphate fraction on DEAE-cellulose, fractions were assayed for starch synthase, branching enzyme and phosphorylase activity. Only a single form of ADPG starch synthase was resolved (Fig. 2A). The starch synthase exhibited both primed activity with amylopectin as glucose acceptor and unprimed activity in the presence of high citrate and bovine serum albumin (BSA). Three branching enzyme isozymes were resolved by DEAE-cellulose chromatography (Fig. 2B). The second peak of activity coeluted with the starch synthase (Fig. 2). No phosphorylase activity was observed in the DEAE-cellulose fractions.

The 500 *g* pellet from differential centrifugation (Fig. 1) was assayed for starch synthase activity using both ADPG and uridine diphosphate glucose (UDPG) as glucose donors (Table 1). Incubation of the pellet fraction for 1 hr with 0.4% CHAPS solubilized 75% of the activities observed with both glucose donors (Table 1). The residual activity may be ascribed to starch granule-

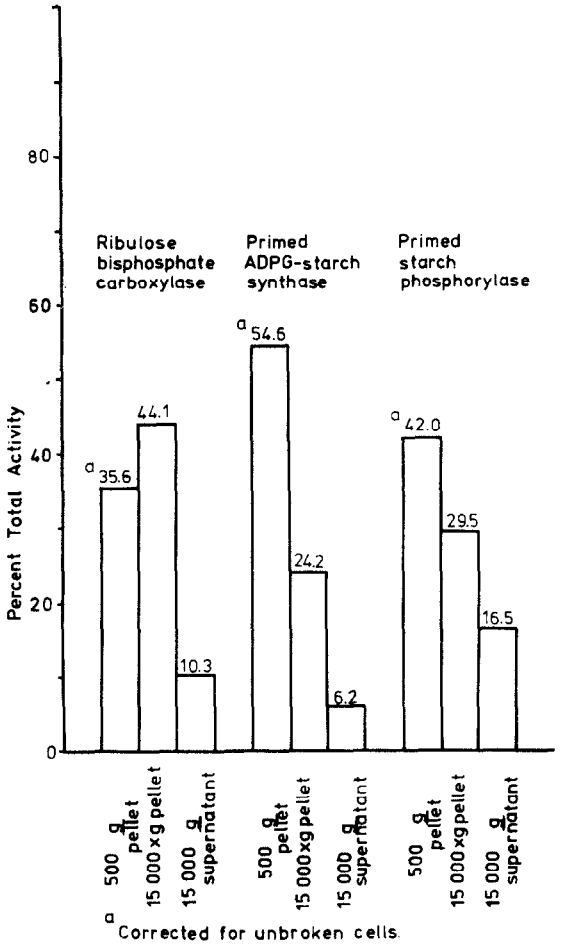

Fig. 1. Distribution of RUBP carboxylase, ADPG starch phosphorylase, and starch phosphorylase from five-day-old castor bean endosperm in pellets obtained by differential centrifugation.

Table 1. Starch synthase activity of plastid-rich pellets of young castor bean endosperm before and after extraction for one hour with 0.4% CHAPS

Glucose donor	Activity nmol glucose incorporated/60 min/50 μl −CHAPS	+CHAPS (Starch granule-bound starch synthase)
ADP-glucose	3.2	0.8
UDP-glucose	0.55	0.2

bound starch synthase. Activity with ADPG was four-fold greater than with UDPG (Table 1).

The activity of the isolated and partially purified starch synthase from the DEAE-cellulose chromatographic preparation was measured under a variety of conditions (Table 2). This form of starch synthase was incapable of utilizing UDPG as a glucose donor in either primed or unprimed (citrate-stimulated) reactions. Starch synthase activity with ADPG as glucose donor was greatest in the

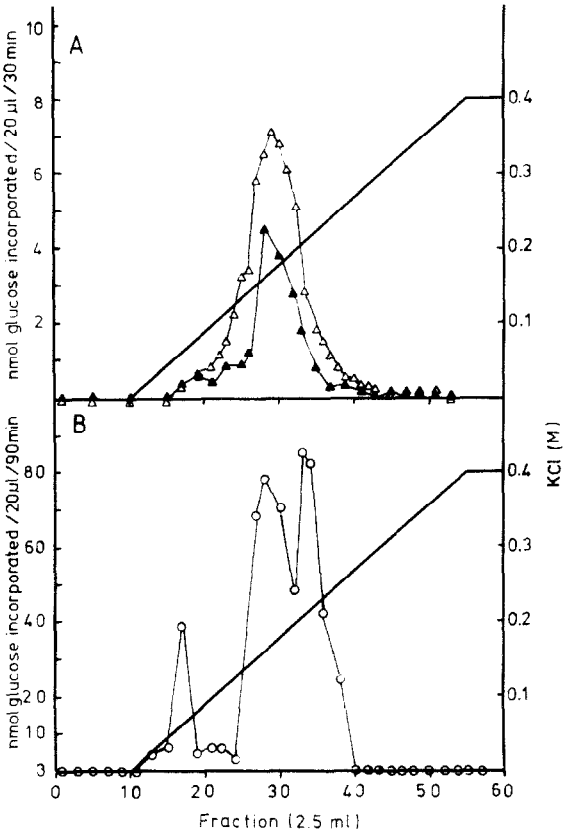

Fig. 2. DEAE-cellulose ion exchange chromatographic profile of ADPG starch synthase and starch branching enzyme from five-day-old castor bean endosperm. A. Primed starch synthase, ▲; citrate-stimulated (unprimed) starch synthase, △. B. Starch branching enzyme, ○.

absence of primer under citrate-stimulated conditions (Table 2). Among the polysaccharide primers, the high M_r rabbit liver glycogen and maize amylopectin are preferred to the lower M_r oyster glycogen (Table 2). Although the rabbit liver glycogen and amylopectin were similar in M_r, the amylopectin, with twice the average chain length of the rabbit liver glycogen, showed 27% less activity than the rabbit liver glycogen. Activity with amylose was very low and pullulan was incapable of serving as a primer for starch synthase (Table 2).

DISCUSSION

ADPG starch synthase and starch phosphorylase were present in plastid-rich pellets of endosperm from five-day-old castor bean seedlings (Fig. 1). Only a single form of ADPG starch synthase with both primed and citrate-stimulated (unprimed) activity was eluted from DEAE-cellulose (Fig. 2A). In other tissues multiple enzyme forms are commonly observed [2–11]; the castor bean enzyme seems most similar to starch synthase I of maize endosperm [13, 14, Goldner and Boyer, in preparation]. Three peaks of branching enzyme activity were resolved by DEAE-cellulose chromatography (Fig. 2B). The second peak coincided with that of starch synthase. In maize endosperm starch synthase I and branching enzyme IIb coelute [3].

Table 2. Effect of different primers on glucosyltransferase activities

Glucan primer*	Activity nmol glucose incorporation/30 min/20 μl ADPG-starch synthase†	Starch phosphorylase‡
0.5 M citrate	4.05	NA§
50 mM glucose 1-phosphate	NA	0.40
14 mM maltose	NA	0.50
Pullulan	0.00	0.80
Amylose	0.65	3.65
Oyster glycogen	1.25	3.00
Amylopectin	2.20	2.35
Rabbit liver glycogen	2.95	2.50

*All primers were obtained from Sigma. Primer concentrations were 5 mg/ml.
†DEAE-cellulose Fr 28 (Fig. 2A) was enzyme source.
‡Enzyme source was the 15 000 *g* supernatant from differential centrifugation (Fig. 1), following gel filtration on Sephadex G-25, and ultrafiltration.
§Not applicable.

As for starch synthase I of maize endosperm [13] and the synthase in maize leaves [15], the activity of the partially purified ADPG starch synthase was greatest in the presence of 0.5 M sodium citrate (no primer added; Table 2). Amylose was found to be a relatively ineffective primer (Table 2), and this observation is similar to that for the maize endosperm enzyme in this respect [16]. The higher M_r branched primers (rabbit liver glycogen) were much more effective (Table 2). For primers of equivalent M_r, those with shorter unit chain lengths (rabbit liver glycogen) were preferred to those with longer unit chain lengths (amylopectin). These responses coincide with those of maize endosperm starch synthase I[17].

The castor bean phosphorylase showed low activity in the absence of primer or with maltose (Table 2). As with the enzymes from other sources [16, 18], the linear potato amylose was more effective than the branched α-glucan primers (Table 2).

The results make it clear that the castor bean endosperm tissue contains all of the enzymatic activities required to make starch from ADPG as well as starch phosphorylase. Furthermore, in the properties examined, the enzymes from castor bean show striking similarities to the corresponding enzymes from tissues that do accumulate starch; no unusual features were observed that would question their function *in vivo*. Thus, in respect of the failure of the castor bean endosperm tissue to accumulate significant amounts of starch when sucrose is present in very high levels it seems that, as suggested by Reibach and Benedict [1], it is indeed the very low activity of the ADPG pyrophosphorylase that must be responsible. A comparable situation has been observed in starchless mutants of *Arabidopsis* [19] and plants which accumulate fructosans, rather than glucans as storage polysaccharides [20] where ADPG pyrophosphorylase is absent, but the activities of the enzymes of starch biosynthesis are present.

EXPERIMENTAL

Plastid-rich pellets of endosperm tissue from 5-day-old dark-germinated *Ricinus communis* L. cv. Hale were produced according to the methods of ref. [1]. Differential centrifugation resulted in three fractions for which enzyme activities were determined. These were the 500 *g* pellet, the 15 000 *g* pellet and the 15 000 *g* supernatant. Additional sepn of organelles was carried out using rate-zonal sucrose density gradients according to ref. [21].

Enzyme assays. The following procedures were used ribulose 1,5-bisphosphate carboxylase [22], alcohol dehydrogenase [23], fumarase [24], ADP-glucose pyrophosphorylase [25]. ADP-glucose starch synthase was assayed following the methods of ref. [11] as described in ref. [13] with some modification. Radiolabelled ADP-glucose sp. act. was 250 cpm/nmol. The reactions were carried out at 34–35° for 30 min in a circulating water bath. Maize amylopectin (Sigma; 5 mg/ml) was boiled for 10–15 min and used as the primer for both the starch synthase assays and the starch phosphorylase assay described below. Pellet frs were resuspended in a minimal vol. of grinding medium without sucrose for the purpose of assaying enzyme activities. Starch phosphorylase was assayed in the direction of synthesis according to the procedure of ref. [18] modified to include the methods of ref. [25] to utilize scintillation counting.

Enzyme isolation and partial purification. Starch synthase and branching enzyme were isolated and partially purified from the endosperm tissue using the methods of ref. [3]. All procedures were carried out at 4°. Endosperm tissue (180 g) from dark-germinated 5-day-old seedlings was homogenized in a minimal amount of 50 mM Tris–acetate buffer (pH 7.5) containing 10 mM EDTA and 2.5 mM DTT in a Waring Blender for 45 sec. The homogenate was filtered through four layers of nylon mesh to remove solids. The filtrate was centrifuged at 35 000 *g* for 15 min. The pellet was discarded and protamine sulphate (2 mg/ml) was added to the supernatant after removal of the upper lipid layer. The soln was centrifuged as above. The pellet was discarded and the supernatant was made 40% satd with $(NH_4)_2SO_4$ and centrifuged. The supernatant was discarded. The pellet was resuspended in buffer and dialysed overnight against 2 l of the same buffer. The dialysed soln was concd by ultrafiltration and applied to a DEAE-cellulose column equilibrated with buffer with 5% sucrose added. Protein was eluted from the column with a one resin-bed vol (135 ml) 0–0.4 mM KCl gradient in elution buffer. Frs (2.5 ml) were collected and assayed for primed and citrate-stimulated (unprimed) starch synthase and branching enzyme activity.

Citrate-stimulated (unprimed) starch synthase activity was assayed by the method of ref. [13] replacing the primer in the

assay procedures described above with an equal vol. of 0.5 M Na citrate. KOAc in the same assay was replaced by an equal vol. of BSA (0.5 mg/ml). Branching enzyme was assayed by the methods of ref. [3]. The basis of the assay is the stimulation by branching enzyme of the synthesis of α-glucan from radiolabelled glucose 1-phosphate catalysed by rabbit muscle phosphorylase. The reaction mixt contained 0.1 M Na citrate (pH 7), 1 mM adenosine monophosphate (AMP), 50 mM [^{14}C]-glucose 1-phosphate (500 cpm/nmol), 40 μg crystalline rabbit muscle phosphorylase a (Sigma) and enzyme fr. in a total vol. of 0.2 ml).

Assay of particulate starch synthase activity. The 500 *g* pellets from the differential centrifugation prepn described above were assayed for incorporation of [^{14}C]-glucose from radiolabelled ADPG (250 cpm/nmol) or UDP-glucose (UDPG; 265 cpm/ nmol) into MeOH insol. polysaccharide. The starch synthase assay was modified to include no primer (glucose acceptor), the difference in the vol of reaction cocktail being replaced by an equal vol of H_2O. Presumably, starch granules present in the pellets could act both as primer and enzyme source. The assay conditions were modified by increasing the time to 60 min. A minimal amount of buffer was used to resuspend the pellets and 0.05 ml aliquots were used in the assay. Solubility of starch synthase activity in the pellets was tested by incubating pellets for 60 min at 30° in the presence of 0.4% {3-[(3-cholamido-propyl) dimethylammonio] 1-propanesulphonate} (CHAPS; Sigma), a mild zwitterionic detergent, in buffer. After 60 min the suspension was centrifuged at 500 *g* for 5 min. The supernatant was used as the source for solubilized enzyme, while the pellet served as source for the particulate activity.

Primer affinity assays. Maize amylopectin, rabbit liver glycogen, oyster glycogen ($M_r > 5 \times 10^6$, 2×10^6, 7×10^5, respectively (Goldner and Boyer, in preparation)), pullulan and potato amylose were obtained from Sigma. All primers were boiled for 10–15 min to disperse the glucans into soln. All primers were used at concns of 5 mg/ml. DEAE-cellulose Fr 28 (Fig. 2A) was used as enzyme source.

The ability of starch phosphorylase to utilize the same set of primers was assayed using the 15 000 *g* supernatant from the differential centrifugation prepn as the enzyme after gel filtration on a Sephadex G-25 column and ultrafiltration.

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