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# SOLUBLE STARCH SYNTHASES AND STARCH BRANCHING ENZYMES FROM DEVELOPING SEEDS OF SORGHUM*

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**Key Word Index**—*Sorghum bicolor*; Gramineae; starch synthesis; ADP-glucose; α-1,4-glucan-4-glucosyltransferase; Q-enzyme.

**Abstract**—Soluble starch synthases and branching enzymes have been partially purified from developing sorghum seeds. Two major fractions and one minor fraction of starch synthase were eluted on DEAE-cellulose chromatography. The minor enzyme eluted first and was similar to the early eluting major synthase in citrate-stimulated activity, faster reaction rates with glycogen primers than amylopectin primers, and in $K_m$ for ADP-glucose (0.05 and 0.08 mM, respectively). The starch synthase peak eluted last had no citrate-stimulated activity, was equally active with glycogen and amylopectin primers, and had the highest $K_m$ for ADP-glucose (0.10 mM). Four fractions of branching enzymes were recovered from DEAE-cellulose chromatography. One fraction eluted in the buffer wash; the other three co-eluted with the three starch synthases. All four fractions could branch amylose or amylopectin, and stimulated α-glucan synthesis catalysed by phosphorylase. Electrophoretic separation and activity staining for starch synthase of crude extracts and DEAE-cellulose fractions demonstrated complex banding patterns. The colour of the bands after iodine staining indicated that branching enzyme and starch synthase co-migrated during electrophoresis.

## INTRODUCTION

Soluble starch synthase (ADP-glucose: α-1,4-glucan-4-glucosyltransferase EC 2.4.1.21) has been separated into multiple forms in extracts from developing maize seeds [1], spinach leaves [2], potato tubers [3], developing seeds of rice [4], peas [5] and teosinte [6] and maize leaves [7]. In general, these studies have demonstrated the ability of one fraction of the soluble starch synthase to catalyse high levels of glucan synthesis in the absence of primer if citrate (0.25–0.5 M) is included in the reaction mixture (citrate-stimulated or unprimed activity); other fractions of soluble starch synthase were only active in reactions containing added primers (primed assays). Multiple forms of starch branching enzyme (α-1,4-glucan: α-1,4-glucan-6-glucosyltransferase, EC 2.4.1.18) have been observed in spinach leaves [2], maize kernels [7, 8], teosinte seeds [6] and pea seeds [5]. The multiplicity of enzymatic reactions is complicated further by the presence of starch synthase tightly bound to starch granules. The starch granule bound starch synthase from maize endosperm starch granules has also recently been separated into two different fractions after solubilization [9].

The role of multiple forms of starch synthases and branching enzymes is difficult to explain. Only two glucosidic linkages are found in the two fractions of the starch molecule. Approximately 96% of the glucosidic bonds are α-1,4 in branched amylopectin molecules with the remaining 4% of the linkages, the branch points, being α-1,6. The linear fraction of starch, amylose, is composed primarily of α-1,4 linkages with an occasional α-1,6 branch (less than 0.5% of the total linkages) [10]. The complexity of the spatial orientation of starch polymers in the starch granule makes assignment of specific functions to different enzyme forms additionally difficult. An exception is the generally accepted role of starch granule bound starch synthase in amylose biosynthesis. Evidence for the role of starch granule bound starch synthase in amylose biosynthesis is based largely on the observation of a deficiency of this enzyme activity in extracts of *waxy* or *glutinous* mutants which contain no amylose (see review [11]).

Work in my laboratory has been directed at the mode(s) of action and roles in starch synthesis of the multiple forms of starch synthase and branching enzyme. To this end, starch structure is being studied, starch synthases and branching enzymes from different species are being described, and genetic variants affecting starch biosynthesis are being characterized. We feel that all three approaches will be required to shed light on starch biosynthesis. In this paper, the properties of soluble starch synthases and branching enzymes from developing seeds of *Sorghum bicolor* are described. Sorghum has a number of properties which make it suitable for the approaches suggested, including abundant tissue (seeds) active in starch biosynthesis and identified mutants affecting starch synthesis [12, 13].

## RESULTS AND DISCUSSION

Soluble starch synthases and starch branching enzymes were purified by procedures previously employed with other enzyme sources (Table 1). Preliminary studies indicated that greater than 75% of the primed starch

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Table 1. Purification of soluble starch synthases and starch branching enzymes from developing sorghum seeds

| Fraction | Volume (ml) | Protein (mg) | Total activity (nkat) | | | Specific activity (nkat/mg) | | |
|---|---|---|---|---|---|---|---|---|
| | | | BE* | Primed | Citrate-stimulated | BE | Primed | Citrate-stimulated |
| Crude | 141 | 774 | 1787 | 20.7 | 12.4 | 2.3 | 0.026 | 0.016 |
| 15000 *g* supernatant | 128 | 191 | 1765 | 17.4 | 8.0 | 9.2 | 0.091 | 0.042 |
| 50% $(NH_4)_2SO_4$ | 28 | 110 | 1618 | 14.7 | 6.0 | 14.7 | 0.134 | 0.054 |
| DEAE-cellulose | | | | | | | | |
| Fraction I (7–25)† | 19.6 | 32.3 | 888 | — | — | 27.5 | — | — |
| Fraction II (34–40) | 11.1 | 6.4 | 132 | 0.4 | 1.3 | 20.6 | 0.062 | 0.203 |
| Fraction III (41–58) | 6.0 | 6.0 | 207 | 1.5 | 2.8 | 34.4 | 0.250 | 0.467 |
| Fraction IV (59–80) | 11.5 | 17.6 | 602 | 3.8 | 0.6 | 34.2 | 0.220 | 0.034 |

*Branching enzyme (assay A).
†Fraction number.

synthase activity and essentially 100% of the branching enzyme remained in the supernatant fraction after centrifugation (Table 1). Precipitation of enzymes with 40% ammonium sulphate effectively complexed starch synthesis activity. However, 30% of the branching enzyme activity remained soluble under these conditions. DEAE-cellulose chromatography identified the branching enzymes remaining soluble at 40% ammonium sulphate as incompletely precipitated fractions I and II described below (data not shown). The concentration of ammonium sulfate is higher than that reported for precipitation of other branching enzymes [2, 5–8]. Ammonium sulfate precipitation at 50% saturation effectively complexed all branching enzymes (Table 1).

Chromatography on DEAE-cellulose of dialysed ammonium sulfate fractions demonstrated multiple fractions of starch synthases and branching enzymes (Fig. 1). Two major fractions of starch synthase eluted from DEAE-cellulose at 0.13 M and 0.17 M KCl, respectively. In addition, a minor fraction of starch synthase eluted earlier in the gradient (0.07 M KCl) than the major fractions (Fig. 1). DEAE-fractions were numbered in the order of elution. Therefore, starch synthase activity was recovered in DEAE-cellulose fractions II, III and IV (Table 1). All four DEAE-cellulose fractions (I–IV) contained branching enzyme activity. Recoveries of primed starch synthase, citrate-stimulated starch synthase and branching enzyme activity after DEAE-cellulose chromatography were approximately 30, 50 and 95%, respectively (Table 1). Increases in specific activity ranged from 10- to 40-fold for all enzymes. After DEAE-cellulose chromatography, the enzymes retained greater than 70% of their activity when stored on ice for up to 1 month. Storage at −20° for 6 months resulted in loss of up to 50% of the original activity.

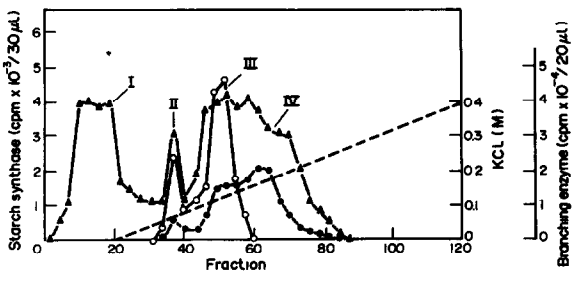


Fig. 1. Elution profiles of DEAE-cellulose chromatography of soluble starch synthases and branching enzymes from *S. bicolor*. (●—●) Primed (amylopectin) starch synthase activity; (○—○) citrate-stimulated starch synthase activity; (▲—▲) branching enzyme activity; (— —) KCl gradient.

Characterization of the starch synthase fractions indicated that the enzymes in fractions II and III were very similar. Both fractions II and III catalysed citrate-stimulated starch synthesis (Table 2). Both enzymes were more active in the primed assay conditions with glycogens then with amylopectin. No detectable incorporation of glucose was measured when ADP-[$^{14}$C]glucose was replaced with UDP-[$^{14}$C]glucose in reactions with either enzyme. Finally, both fractions II and III had similar $K_m$s for ADP-glucose (Table 2). Starch synthase fraction IV had very low citrate-stimulated activity, most likely due to incomplete separation of fraction III enzyme (Tables 1 and 2). In addition, nearly equal primed activity was observed with amylopectin or glycogen in the reaction mixture (Table 2). In primed reactions, fraction IV incorporated glucose from UDP-[$^{14}$C]glucose at 6% the rate observed for reactions with ADP-[$^{14}$C]glucose. In summary, sorghum seed, as with other species [1–7], contains multiple forms of starch synthase with at least one fraction capable of citrate-stimulated synthesis and higher activity with glycogen primers than amylopectin.

The multiple forms of branching enzymes were further characterized. All four enzyme fractions were capable of branching amylose and amylopectin as measured by changes in absorbance of the iodine–glucan complex (Table 3). The branching of amylose was complete within 2 hr and no further change was observed after 24 hr (not shown). The branching of amylopectin, however, required up to 24 hr for completion. The ratios of branching enzyme activity measured by phosphorylase a stimulation (assay A) divided by the activity measured by amylose branching (assay B) ranged from 5 to 35 (Table 3).

Based on the results presented above, the pattern of soluble starch synthases and branching enzymes from developing sorghum seeds is similar to patterns reported in previous studies [1–7]. An increasing body of evidence

Table 2. Properties of sorghum starch synthase fractions from DEAE-cellulose chromatography

| Property | DEAE-cellulose fraction II | III | IV |
|---|---|---|---|
| Relative activity* | | | |
| Amylopectin | 100 | 100 | 100 |
| Rabbit liver glycogen | 301 ± 38 | 309 ± 14 | 139 ± 17 |
| Oyster glycogen | 210 ± 12 | 222 ± 18 | 103 ± 8 |
| $K_m$ ADPG (mM) | 0.05 | 0.08 | 0.10 |
| Ratio unprimed activity/primed activity* | | | |
| Amylopectin | 2.66 | 2.50 | 0.08 |
| Rabbit liver glycogen | 0.88 | 0.81 | 0.06 |

*Primed assays: All assays contained 5 mg/ml primer. Activity of assays containing amylopectin was arbitrarily set at 100.

Table 3. Properties of sorghum branching enzyme fractions from DEAE-cellulose chromatography

| Branching enzyme fraction | Ratio assay A/ assay B | $\lambda_{max}$* (nm) Amylose −BE | Amylose +BE | Amylopectin −BE | Amylopectin +BE |
|---|---|---|---|---|---|
| I | 10 | 620 | 530 | 540 | 460 |
| II | 5 | 620 | 540 | 540 | 460 |
| III | 8 | 620 | 560 | 540 | 460 |
| IV | 35 | 620 | 560 | 540 | 460 |

*Absorbance maxima of iodine–glucan complex after 24 hr in the presence (+BE) or absence (−BE) of indicated branching enzyme fraction.

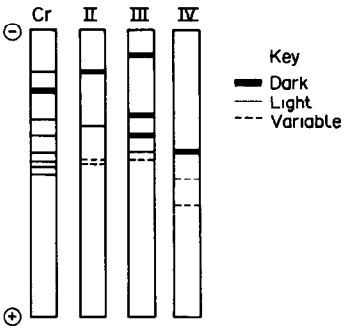


Fig. 2. Zymogens of sorghum starch synthases in DEAE-cellulose fractions II, III and IV and crude extracts (Cr) from sorghum seeds. Procedures are described in the Experimental.

leads me to suggest that multiple forms of these enzymes are the rule not the exception. Multiple forms of starch synthases have been found in maize seeds and leaves [1, 7], spinach leaves [2], potato tubers [3], and developing seeds of rice [4], peas [5] and teosinte [6]. Multiple forms of branching enzyme have been reported in spinach leaves [2] and seeds of maize [7, 8], teosinte [6] and peas [5]. A single fraction of starch synthase has been reported in grape leaves [7], and a single branching enzyme was described in potato tubers [14]. These two cases are presently the only reports of the isolation of single enzyme fractions. In particular, the sorghum enzymes are very similar to the enzymes from developing maize seeds. With the exception of fraction II, which has not been observed in extracts from maize, the properties of the sorghum enzymes are nearly equivalent to the comparable maize enzymes. As maize and sorghum are closely related, this is not necessarily surprising. Electrophoresis of crude extracts from developing maize seeds followed by activity staining for starch synthase typically demonstrates up to nine distinct bands [15]. However, electrophoretic patterns of purified fractions of the maize starch synthases generally are simpler as well as contain additional bands not seen in the patterns of crude extracts [16]. Electrophoretic separations of crude extracts and purified sorghum starch synthase are very similar (Fig. 2). Some of the activity bands observed in the crude extracts can be observed in different enzyme fractions; other bands cannot (Fig. 2). Bands not observed in crude extracts are present in all three enzyme fractions. Glucan produced by slower bands stained purplish and faster moving bands produced glucan staining brown. No glucan bands stained blue, indicating that branching enzyme was co-migrating with the starch synthases. Gels stained for protein demonstrated the presence of many non-synthase proteins in the fractions.

The similarities between the sorghum enzymes and maize enzymes lead me to suggest that sorghum mutations affecting starch biosynthesis in the seed might be found and proven homologous to the large number of maize mutants now known [11]. To date, two such mutants of sorghum have been identified [12, 13] and these appear homologous to *waxy* and *sugary* mutants of maize [11, 12, 17]. The diploid genetic makeup of sorghum should make the search for addition mutants in the starch synthesis pathway feasible. We are currently developing mutation breeding experiments for this purpose.

## EXPERIMENTAL

*Materials.* Sorghum plants (*S. bicolor* cv M5186; Pfizer Genetics) were grown in the greenhouse during the spring of 1982. Heads were bagged and shaken to ensure self-pollination.

Seeds were harvested at 22 days after pollination (midfill period), quick-frozen and stored at −20° until used. [$^{14}$C]Glucose-1-phosphate, ADP-[$^{14}$C]glucose and UDP-[$^{14}$C]glucose were purchased from Amersham/Searle. Potato amylose, crystalline rabbit muscle phosphorylase a, rabbit liver glycogen type III and oyster glycogen were obtained from Sigma. Potato amylopectin was obtained from U.S. Biochemicals.

*Enzyme assays.* ADP-glucose: starch synthase: The incorporation of [$^{14}$C]glucose from ADP-[$^{14}$C]glucose into primer (5 mg/ml, primed assay) or MeOH-insoluble glucan in reactions containing 0.5 M citrate but no added primer (citrate-stimulated assay) was determined as described in ref. [2]. In order to determine nucleotide specificity, ADP-[$^{14}$C]glucose was replaced by UDP-[$^{14}$C]glucose in primed reaction mixtures. Branching enzyme: The branching enzyme stimulation of [$^{14}$C]glucan formation from [$^{14}$C]glucose-1-phosphate catalysed by phosphorylase a (assay A) was determined as described in ref. [2]. The change in absorbance at 660 nm of the amylose–$I_2$ complex (assay B) was followed as described in ref. [8]. In some experiments, amylose was replaced by amylopectin, 10 mg/ml.

*Enzyme purification.* All procedures were carried out at 0–4°. Following homogenization, the resulting extract was filtered through Miracloth. The crude extracts were purified by centrifugation (30 min at 15000 *g*), $(NH_4)_2SO_4$ precipitation (50%) and DEAE-cellulose chromatography. A detailed description of buffers and procedures has been described [18]. Enzymes were routinely purified from 20–40 g of seeds.

*Protein.* Protein was measured as described in ref. [19] using bovine serum albumin as a standard.

*Electrophoresis.* PAGE was performed by a modification of the procedure of Davis [20]. Running gels (7.5%) were prepared as described with the addition of amylose (30 μg/ml). Stacking gels contained no amylose and other solns were unchanged. After electrophoresis, gels were removed and incubated in a soln containing 1 mM ADP-glucose, 30 mM GSH, 20 mM EDTA, 75 mM citric acid and 50 mM Bicine, pH 8.0, for 24 hr [15]. Glucan formation was detected by staining with KI, $I_2$ [21].

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