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STARCH SYNTHASES AND STARCH BRANCHING ENZYMES FROM *PISUM SATIVUM*

GAIL L. MATTERS and CHARLES D. BOYER

Department of Horticulture and Forestry, Rutgers University, P.O. Box 231, New Brunswick, NJ 08903, U.S.A.

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Key Word Index—*Pisum sativum*; Leguminosae; pea; starch synthesis; ADPglucose; α-1,4-glucan-4-glucosyltransferase; Q-enzyme; branching enzyme; isoenzymes.

Abstract—Concentrations of ADPglucose:α-1,4-glucan-4-glucosyltransferase (starch synthase) and α-1,4 glucan:α-1,4-glucan-6-glycosyltransferase (branching enzyme) from developing seeds of *Pisum sativum* were measured. Primed starch synthase activity increased from 8 to 14 days after anthesis and decreased by 50% at 26 days. Citrate-stimulated starch synthase activity was highest at 10 days after anthesis decreasing to low levels by 22 days. Branching enzyme activity increased from 8 to 18 days after anthesis and decreased little by 26 days. Two fractions of starch synthase were recovered by gradient elution from DEAE-cellulose of extracts from 12- and 18-day-old seeds. The two fractions differed in primer specificity, K_m for ADPG and relative amounts of citrate-stimulated activity. A major and minor fraction of branching enzyme were observed in extracts from both 12- and 18-day-old seeds. Marked differences in the relative abilities of the two branching enzyme fractions to stimulate phosphorylase and to branch amylose as well as pH optima were found. Although the content of the starch synthase and branching enzyme fractions varied with seed age, little difference was seen in the properties of chromatographically similar fractions. Therefore, the changes in starch synthase and branching enzyme activity during pea seed development resulted from changes in the concentrations of a few enzyme forms, but not the appearance of different enzyme forms.

INTRODUCTION

Multiple forms of soluble starch synthase (ADPglucose:α-1,4-glucan-4-glucosyltransferase, EC 2.4.1.21) have been isolated from several sources including maize kernels [1], spinach leaves [2], rice seeds [3], and potato tubers [4]. One of the forms in each tissue catalysed glucan synthesis in the absence of added primer and the presence of 0.5 M citrate (citrate-stimulated starch synthesis). The other fractions, however, required primer to be active. Branching enzyme (α-1,4-glucan: α-1,4-glucan-6-glucosyltransferase, EC 2.4.1.18) has been separated into multiple forms in extracts from maize kernels [5] and spinach leaves [2]. In addition to forming the branch points in the amylopectin component of the starch granule, by branching growing starch molecules, branching enzymes increased the citrate-stimulated starch synthase reaction [2, 6].

The elucidation of the roles of the multiple forms of starch synthase and branching enzyme in the biosynthesis of starch requires molecular characterization of each fraction. Furthermore, changes in the enzyme fractions during development should provide insight into the possible role of the multiple enzyme forms as well as the developmental processes in the tissue in question. Although the levels of starch biosynthetic enzymes have been shown to change with tissue age [7], studies have not been directed at the possible changes in the enzyme fractions during development. The present work was undertaken with this objective in mind. Concentrations of starch synthase and branching enzyme during development of pea seeds have now been measured.

RESULTS

Starch synthase and starch branching enzyme activities were detected in crude extracts as early as 8 days after anthesis (Fig. 1). Primed starch synthase activity was highest from 10 to 26 days after anthesis and peaked at 14 days. At all developmental stages, primed synthase

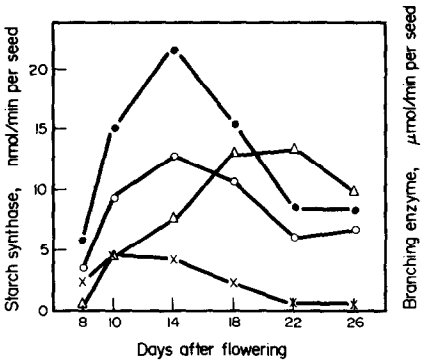

Fig. 1. Starch synthase and branching enzyme activity in extracts from developing pea seed at different times after anthesis. Primed starch synthase activity (rabbit liver glycogen, 5 mg/ml), ●; primed starch synthase activity (amylopectin, 5 mg/ml), ○; citrate-stimulated starch synthase activity, × ; branching enzyme activity, △.

activity was higher when rabbit liver glycogen was used as primer than when amylopectin was used. The citrate-stimulated starch synthase activity, although present in low amounts, was highest at 10–14 days after anthesis and fell to very low concentrations by 22 days. Branching enzyme activity rose from 8 to 18 days after anthesis and remained high to 26 days (Fig. 1).

In order to compare the enzymes during development, starch synthase and starch branching enzyme were partially purified from 12- and 18-day-old seed. The enzyme activities during the stages of purification are given in Tables 1 and 2. DEAE-cellulose chromatography profiles of 12- and 18-day-old extracts showed two peaks of starch synthase activity (Fig. 2). Peaks eluted in the KCl gradient at salt concentration of 0.05 and 0.08 M in both extracts. In the 12-day-old sample, both starch synthase peaks showed citrate-stimulated starch synthesis. In contrast, the DEAE-cellulose profile from the 18-day-old sample exhibited only one peak of citrate-stimulated activity (Fig. 2). However, after concentration, the second fraction of starch synthase also showed citrate-stimulated activity (Table 2). In addition, the citrate-stimulated synthase activity was greater in concentrated fraction I from 18-day-old extracts than anticipated from the column profile.

DEAE-cellulose elution profiles showed a major peak of branching enzyme activity (Fig. 2). As expected from the developmental studies, the quantity of branching enzyme was greater in 18-day-old samples than in 12-day-old samples. A second fraction of branching enzyme was observed as a shoulder overlapping the latter eluting starch synthase peak. All three fractions recovered from DEAE-cellulose chromatography contained amylolytic activity as seen by the release of reducing sugars from amylopectin. Glucose and maltose were identified by PC as the major sugars released.

The citrate-stimulated starch synthase activity in concentrated DEAE-cellulose fractions decreased when stored at 4° (Table 3). During the same period, the primed synthase activity changed little. Citrate-stimulated activity could be regenerated by the addition of rabbit liver glycogen (final concentration 1 mg/ml) to an aliquot of the fraction (Table 3). At this concentration, citrate-stimulated reaction mixtures contained 5–20 μg/ml of rabbit liver glycogen, which is below the concentration required to detect the prime reaction. Further storage at 4° of the mixture of enzyme and rabbit liver glycogen resulted in loss of the regenerated citrate-stimulated activity. The primed starch synthase activity became unstable only after the citrate-stimulated activity was lost.

The two DEAE-cellulose fractions of starch synthase showed differences in reaction velocity in assays containing various primers (Table 4). At 12 days, both synthase fractions had similar relative activities for the different primers tested. Rabbit liver glycogen was the most effective primer, followed by *E. coli* glycogen, oyster glycogen and amylopectin. However, primer specificity differed between starch synthase fractions I and II from 18-day-old samples. Fraction II showed the same order and relative activity for primers in 12-day-old and 18-day-old samples. In comparison, in 18-day-old extracts fraction I starch synthase was most active with rabbit liver glycogen but more active with amylopectin than oyster glycogen (Table 4). The K_m for ADPG was 100 μM for fraction I and 240 μM for fraction II.

Little differences were observed between fractions of branching enzyme from 12-day-old and 18-day-old samples. All branching enzymes were stable for several months at 4°. When assayed by phosphorylase a stimulation, 90% of the total branching enzyme activity recovered from DEAE-cellulose chromatography was found in fraction III. However, when assayed by the branching of amylose, the branching enzyme activity was equally distributed in DEAE-cellulose fractions II and III. The ratio of activity (phosphorylase a stimulation units/amylose branching units) was 4–8 for fraction II and 40–50 for fraction III. Amylose branching was characterized by a linear fall in the absorbance at 660 nm and a drop in the wavelength maximum from 650 to 580 nm of the iodine–amylose complex over a 30 min period.

Further purification of both fraction II and III from 18-day-old seed by aminobutyl Sepharose chromatography reduced amylolytic activities. Although the ratios of phosphorylase a stimulation activity to amylose branching activity changed to 40–80 and 400–500 for fractions II and III, respectively, the two fractions clearly remained different. Similarly, the pH optimum in the

Table 1. Purification of soluble starch synthases and branching enzymes from 12-day-old pea seed

Fraction	Volume (ml)	Protein (mg)	Total activity (units)*			Specific activity (units/mg)		
			BE†	Unprimed	Primed	BE	Unprimed	Primed
Crude	29.5	132.8	30.8	3.7	9.7	0.23	0.027	0.073
10000 g supernatant	27.0	62.2	21.8	3.0	4.7	0.36	0.049	0.076
0–40% $(NH_4)_2SO_4$	16.0	16.3	31.8	2.2	5.0	1.94	0.13	0.31
DEAE-cellulose								
Fraction I (18–25)‡	5.0	0.35	0.22	0.75	0.75	0.63	2.15	2.15
Fraction II (26–36)	5.0	1.51	1.1	1.0	1.4	0.73	0.67	0.93
Fraction III (37–49)	7.0	3.20	14.1	0	0.33	4.41	0	0.10

* One unit of starch synthase is expressed 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.

† Branching enzyme.

‡ Fraction number.

Table 2. Purification of soluble starch synthases and branching enzymes from 18-day-old pea seed

Fraction	Volume (ml)	Protein (mg)	Total activity (units)*			Specific activity (units/mg)		
			BE†	Unprimed	Primed	BE	Unprimed	Primed
Crude	128	555	203	2.1	14.4	0.37	0.0037	0.025
10000 g supernatant	119	268	200	2.3	13.6	0.84	0.0087	0.052
0–40% $(NH_4)_2SO_4$	22	115	97	1.4	13.5	0.83	0.012	0.12
DEAE-cellulose								
Fraction I (46–65)‡	10.3	7.34	4.0	3.8	4.0	0.54	0.52	0.55
Fraction II (66–89)	12.8	3.71	17.4	5.0	4.6	4.7	1.43	1.2
Fraction III (90–125)	11.3	4.16	0.14	0	1.4	27.3	0	0.38

* Enzyme units as described in Table 1.

† Branching enzyme.

‡ Fraction number.

amylose branching assay for the two fractions of branching enzyme differed. Fraction III branching enzyme was almost twice as active in citrate buffer as phosphate buffer with a broad pH optimum range from 6 to 8. Fraction II branching enzyme was equally active in phosphate and citrate buffers, but also showed activity over a broad pH range with activity being highest at pH 7.

DEAE-cellulose fraction III branching enzyme eluted as two overlapping peaks from aminobutyl Sepharose (Fig. 3). These peaks could not be distinguished by any of the assay procedures used. Disc-gel electrophoresis in the presence of amylose for activity staining also showed two bands of activity for fraction III branching enzyme.

DISCUSSION

Based on previous studies [1–5], the presence of multiple forms of starch synthases and branching enzyme in developing pea seeds was expected. The overall elution profile of the enzymes from DEAE-cellulose was similar to the DEAE-cellulose profiles of starch synthases and branching enzymes from spinach leaves [2]. Unlike the starch synthases of the seeds of maize [1] and rice [3], both fractions of starch synthase in peas showed citrate-stimulated activity. Although the citrate-stimulated activity was not detected in all DEAE-cellulose column fractions showing primed starch synthase activity, citrate-

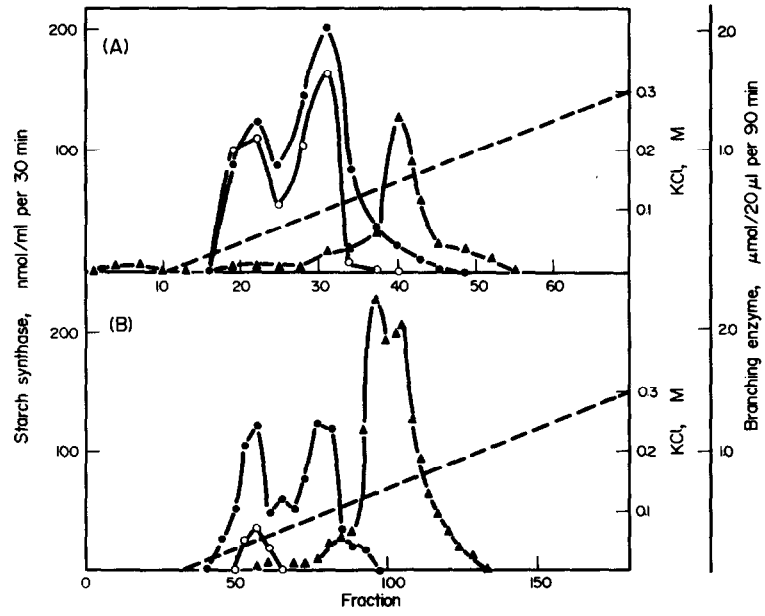

Fig. 2. Elution profiles from DEAE-cellulose of starch synthases and branching enzymes from 12-day-old pea seed (A) and 18-day-old pea seed (B). Primed (amylopectin) starch synthase activity, ●; citrate-stimulated starch synthase activity, ○; branching enzyme activity, ▲. Dashed line shows position of the KCl gradient.

Table 3. Effect of storage and low concentrations of exogenous primer on the citrate-stimulated and primed reactions of starch synthase I from 18-day-old seed

Enzyme condition	[^{14}C]-Glucose transferred (nmol)	
	Primed	Unprimed
DEAE-cellulose I	74.6	50.1
After 10 days at 4°	73.1	44.6
After 20 days at 4°	65.0	1.6
After 20 days at 4°		
+RLG* (1 mg/ml)	—	40.3
+RLG (10 days at 4°)	—	1.8

*RLG: rabbit liver glycogen.

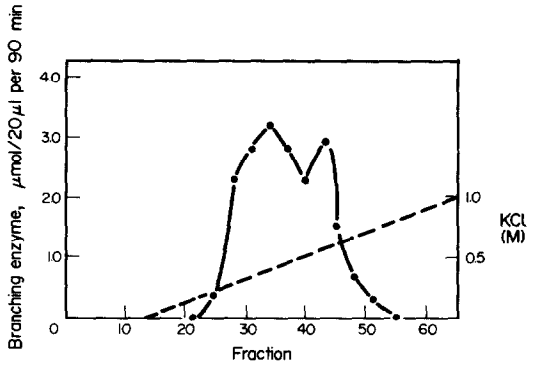

Fig. 3. Purification of branching enzyme DEAE-cellulose fraction III from 18-day-old pea seed by aminobutyl Sepharose chromatography.

stimulated activity was recovered in all concentrated fractions.

The failure to detect citrate-stimulated activity in the DEAE-cellulose column fractions in 18-day-old samples could result from several factors. First, the starch synthase could no longer be capable of citrate-stimulated starch synthesis. Second, at later stages of development, less soluble endogenous primer might be present. Therefore, at 18 days, concentrations of endogenous primer in column fractions may be insufficient to detect citrate-stimulated activity. Finally, amylolytic enzymes may compete with the citrate-stimulated reaction and/or eliminate primer from the fractions. The detection of citrate-stimulated activity in concentrated fractions eliminates the first possibility. Furthermore, concentration of column fractions would also concentrate degrading enzymes as well as endogenous primer. Because concentrated fractions had citrate-stimulated activity, the direct effect of degrading enzyme on the citrate-stimulated reaction would seem unlikely. Therefore, the amounts of endogenous primer associated with the starch synthase fractions would be the remaining factor to consider. The possible effects of endogenous primer concentration are further delineated by the behavior of the starch synthase when stored at 4°. Although the measurable citrate-stimulated activity is lost during storage, the ability of the starch synthases to use low concentrations of primer in the presence of citrate was still present, as demonstrated by the addition of rabbit liver glycogen (Table 3). The citrate-stimulated starch synthase reaction has been shown to result from the lowering of the K_m for primer [2, 6]. As such, the reaction does not appear to be *de novo* but depends on endogenous primer. Therefore, the observed pattern of citrate-stimulated activity on DEAE-cellulose columns could be explained by low endogenous primer concentrations. For example, concentration of column fractions increased the primer concentrations to a level sufficient for citrate-stimulated synthesis. Furthermore, storage with associated degrading enzymes could have resulted in loss of primer and measurable citrate-stimulated activity even though the ability to respond to citrate was still present. Therefore, the differences in the amounts of citrate-stimulated starch synthase activity in 12- and 18-day-old extracts may not reflect differences in the enzymes *per se*.

Table 4. Primed activity of starch synthases from 12- and 18-day-old pea seed.

Primer*	DEAE-cellulose fraction			
	I		II	
	12 days old	18 days old	12 days old	18 days old
Amylopectin	100†	100	100	100
Rabbit liver glycogen	203	169	217	208
Oyster glycogen	119	76	119	123
E. coli glycogen	171	n.d.‡	184	n.d.

*All final primer concentrations were 5 mg/ml.
†Percentage of rate observed with amylopectin.
‡n.d. = Not determined.

With the exception of the citrate-stimulated activity in 18-day-old extracts, the recoveries of enzyme activities after purification were not different from levels predicted with crude extracts (Fig. 1). Although the multiple forms of starch synthase and branching enzyme had differing properties, little differences were found in the properties of the same starch synthase and branching enzyme fractions from 12- and 18-day-old samples. Therefore, the changes in enzyme activity during development appear to result from changes in concentrations of a few enzymes present during the synthetic stage of development and not the appearance of unique enzyme forms.

EXPERIMENTAL

Materials. Pea plants (*Pisum sativum* cv Alaska) were grown in growth chambers at 21°. Seed was harvested at 8–26 days post-anthesis and stored at −5° until used. Glucose [^{14}C]-1-phosphate was purchased from Amersham/Searle. ADP-[^{14}C]glucose was a gift from Dr. J. Preiss, University of California Davis. Potato amylose, crystalline rabbit muscle phosphorylase a and rabbit liver glycogen Type III were obtained from Sigma. Potato amylopectin was purchased from U.S. Biochemicals.

Developmental study. Seeds (1–3 g) were ground in a chilled mortar and pestle in cold 100 mM citrate buffer, pH 7, containing

5 mM DTE. The homogenate was assayed directly. Enzyme activities per seed were determined from 2 replicates.

Enzyme assays. ADPglucose:starch synthase: Incorporation of [^{14}C]glucose into primer, 5 mg/ml (primed activity) or MeOH insoluble glucan in reactions containing 0.5 M citrate without added primer (citrate-stimulated activity) were determined at 30° as described in ref. [2]. Branching enzyme: The stimulation of [^{14}C]glucan formation from glucose-1-P by phosphorylase a (phosphorylase stimulation) was determined as described in ref. [2]. The change in the absorption spectra of amylose–I_2 complex (amylose branching) was followed in reactions as described in ref. [5]. Amylolytic enzymes: The release of reducing sugars from amylopectin at 30° was followed by the Cu method [8]. Reducing sugars were identified by PC using *n*-BuOH–pyridine–H_2O (6:4:1).

Enzyme purification. All operations were carried out at 0–4°. After homogenization, crude extracts were purified by centrifugation (30 min at 10000 g), $(NH_4)_2SO_4$ precipitation (40% satn) and DEAE-cellulose chromatography as described in ref. [5]. Enzymes were purified from 6.1 g of 12-day-old seed and 55.1 g of 18-day-old seed.

Protein was measured by the procedure of ref. [9] using BSA as a standard.

Electrophoresis. Polyacrylamide disc gel electrophoresis was run by a modification of the procedure of ref. [10]. Running gels (7.5%) were polymerized in the presence of amylose (400 mg/ml). The remainder of the solns were unchanged. After electrophoresis, gels were removed from the tubes, incubated in 100 mM citrate buffer (pH 7) containing 5 mM DTE for 2–24 hr and stained for activity with I_2 [11].

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