Phytochemistry, Vol. 22, No. 7, pp. 1573–1576, 1983.
Printed in Great Britain.

0031–9422/83 $3.00 + 0.00

ENZYMES INVOLVED IN STARCH SYNTHESIS IN THE DEVELOPING MUNG BEAN SEED

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(*Revised received* 17 *June* 1982)

Key Word Index—*Vigna radiata*; Leguminosae; sucrose synthetase; starch synthetase; phosphorylase; ADP and UDP glucose pyrophosphorylase; soluble sugars content; starch content.

Abstract—The levels of free sugars, starch and enzymes involved in starch metabolism—sucrose synthetase, UDP and ADP glucose pyrophosphorylase, phosphorylase and starch synthetase—were assayed during seed development of three cultivars of mung bean (*Vigna radiata*). Free sugars and starch increased with increasing seed weight. Changes in levels of sucrose synthetase, UDP- and ADP-glucose pyrophosphorylases, and phosphorylase were paralleled by changes in starch accumulation. After the maximum activity levels of these enzymes had been reached, maximum activities of soluble starch synthetase and starch granule-bound starch synthetase occurred. There were high activities of sucrose synthetase and phosphorylase at maximum rates of starch accumulation. Thus, starch could be synthesized via the ADP glucose pathway in mung bean seeds. However, phosphorylase may account for the starch accumulation in the early stages of mung bean seed development.

INTRODUCTION

Carbohydrate is the major constituent (*ca* 70%) of the mung bean seed [1] and an important factor determining seed yield. Starch metabolism seems to be of particular importance in the early stages of mung bean seed development, since most photosynthates produced in the reproductive phase will be used in reproductive growth [2]. Qualitative and quantitative changes in sugars and starch have been studied in other leguminous seeds at the developing stage [3]. However, information on mung bean (*Vigna radiata*) is marginal. In this paper, we have measured various enzymes related to starch synthesis at different seed filling stages in three mung bean cultivars and have attempted to relate these to starch accumulation.

RESULTS

Growth and carbohydrates of the developing seed

Changes in dry wts and carbohydrates during seed development are shown in Table 1. Dry wts of all cultivars increased little during the first 7 days, but rose rapidly afterward to attain a maximum at *ca* 13 days after flowering (DAF), after which the dry wt changed slightly.

The free sugar content per seed of all three cultivars increased from the first sampling until *ca* 13 DAF, after which it started to decrease. As a percentage of dry wt the free sugar content increased between 5 and 7 DAF for CPI-39755A, but did not change greatly for the other two cultivars. The starch content per seed was minimal at the first sampling (5 DAF). However, the maximum rate of increase in starch content per seed occurred between 7 and 11 DAF in all three cultivars. Starch content per seed started to decrease at 13 DAF in ML-6, whereas in CES-55 and CPI-30755A there was little change after 13 DAF.

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Activity of various enzymes during seed development

Activity of sucrose synthetase was barely detectable at 5 DAF (Fig. 1). From this point, activity increased rapidly, reaching a peak at 11 DAF in ML-6 and CPI-30755A, and at 13 DAF in CES-55, and then declining through 15 DAF.

Activities of UDP- and ADP-glucose pyrophosphorylases are shown in Fig. 2. UDP-glucose pyrophosphorylase activity was very low between 5 and 9 DAF, but increased rapidly after 9 DAF in all three cultivars. Activity reached a maximum at 11 DAF in CPI-30755A, and at 13 DAF in CES-55, after which it declined. Such activity in ML-6 reached a maximum at 11 DAF and remained at a constant level until 15 DAF. Both ML-6 and CES-55 registered a steady increase of ADP-glucose pyrophosphorylase activity starting from the beginning of seed development. Activity of ADP-glucose pyrophosphorylase in CPI-30755A was relatively low throughout the course of seed development.

Activity of phosphorylase at 11 or 13 DAF was 8–10-fold greater than was observed at 8 DAF (Fig. 3). The level of phosphorylase at 11 or 13 DAF in ML-6 was *ca* 60–70% of that found in CES-55 and CPI-30755A.

The activity of soluble starch synthetase in CES-55 and CPI-30755A increased rapidly at 11 DAF, whereas in ML-6 it increased slowly until 15 DAF (Fig. 4). The activity of starch granule-bound starch synthetase in all three cultivars increased slowly and remained at a low level throughout the course of seed development.

DISCUSSION

The maximum rates of free sugar accumulation (as a percentage) in all three cultivars were observed at 5–7 DAF, which was before the maximum rates of starch accumulation. Translocated sucrose is the main raw material for the synthesis of starch in the developing seed and it is the principal free sugar during seed develop-

Table 1. Changes in dry wt and carbohydrates at different stages of mung bean seed development

Cultivars	Days after flowering	Dry wt (mg/seed)	Sugar (mg/seed)	Sugar (% dry wt)	Starch (mg/seed)	Starch (% dry wt)
ML-6	5	0.5	0.02	4.0	0.01	2.0
	7	2.6	0.16	6.2	0.19	7.3
	9	7.7	0.45	5.8	1.07	13.9
	11	30.9	1.97	4.6	11.41	36.9
	13	44.2	2.34	5.3	18.97	42.9
	15	40.7	2.27	5.6	15.86	39.0
	s.e.	12.8	0.71	0.49	5.52	11.6
CES-55	5	0.5	0.02	4.0	0.02	4.0
	7	2.3	0.10	4.3	0.15	6.5
	9	10.6	0.70	6.6	2.05	19.3
	11	40.3	2.59	6.4	12.96	32.2
	13	60.1	3.30	5.5	23.26	38.7
	15	60.5	3.28	5.4	24.67	40.8
	s.e.	18.2	1.01	0.71	7.42	10.4
CPI-30755A	5	0.5	0.01	2.0	0.02	4.0
	7	5.0	0.38	7.6	0.71	14.2
	9	20.6	1.09	5.3	6.38	31.0
	11	44.7	2.37	5.3	18.09	40.5
	13	48.6	2.42	5.0	20.87	42.9
	15	56.1	2.24	4.0	21.75	38.8
	s.e.	15.3	0.69	1.20	6.52	10.2

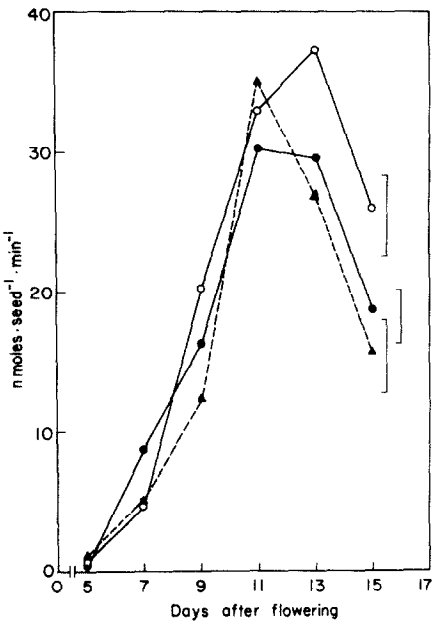

Fig. 1. Changes in the activities of sucrose synthetase in developing mung bean seeds of ML-6 (▲), CES-55 (○) and CPI-30755A (●). Vertical bars represent s.e.

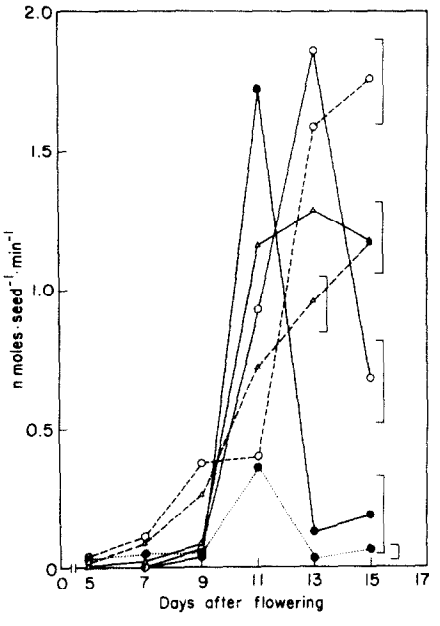

Fig. 2. Changes in the activities of ADP glucose pyrophosphorylases (· · · ·) and UDP-glucose pyrophosphorylase (——) in developing mung bean seeds of ML-6 (△), CES-55 (○) and CPI-30755A (●). Vertical bars represent s.e.

ment [3]. The levelling of the free sugar accumulation at 13–15 DAF in all three cultivars probably reflects the end of sucrose translocation into the seed, at which time the rate of starch accumulation also began to level. In addition, the cultivar with the largest seed (CES-55) had a slightly higher level of free sugars at 11–15 DAF than the other two cultivars. The direct relationship between the concentration of free sugar in the seed and the rate of starch synthesis may suggest that synthesis of starch is regulated by the supply of assimilates to the seed. The rate of rice grain development and dry matter accumulation also was directly affected by the sucrose level [4].

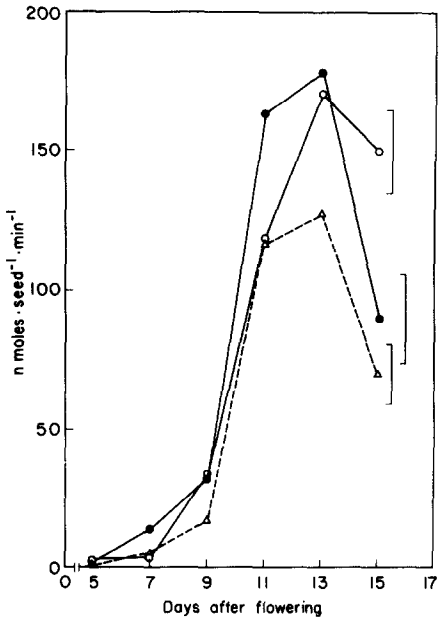

Fig. 3. Changes in the activities of phosphorylase in developing mung bean seeds of ML-6 (△), CES-55 (○) and CPI-30755A (●). Vertical bars represent s.e.

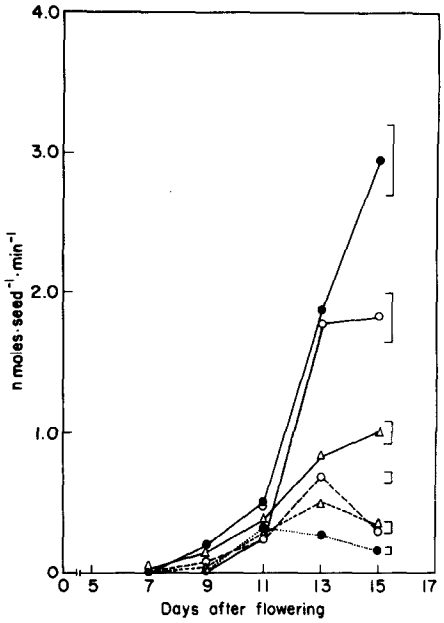

Fig. 4. Changes in the activities of soluble (——) and starch granule-bound (· · · ·) starch synthetase in developing mung bean seeds of ML-6 (△), CES-55 (○) and CPI-30755A (●). Vertical bars represent s.e.

However, other investigations [5] have indicated that the normal pattern of accumulation of dry matter in the seed is determined within the seed itself. Termination of the accumulation of starch as the seed matures could, therefore, conceivably be due to either the loss of synthetic capacity of the seed, or to cessation of the supply of assimilates to the seed, or both [6].

Cleavage of sucrose can be achieved by invertase, but it cannot account for the metabolism of sucrose in the seed [6]. Instead, sucrose transported from leaves is converted to UDP-glucose by the action of sucrose synthetase and liberated UDP-glucose is converted to glucose-1-phosphate via UDP-glucose pyrophosphorylase. The glucose-l-phosphate produced would be available for ADP-glucose formation by ADP-glucose pyrophosphorylase or utilized directly for starch synthesis by phosphorylase [3, 7]. The concomitant increase of sucrose synthesis and free sugars and starch suggests that sucrose synthetase catalyses the first step in the conversion of sucrose to starch. The results showed that sucrose synthetase is negligible at 5 DAF when only a small amount of starch is synthesized. Sucrose synthetase in the large seeded CES-55 has an activity rate similar to those in the other two cultivars until 11 DAF, after which the activities in ML-6 and CPI-30755A declined. The activity in CES-55, however, did not decline until 15 DAF. This may reflect the continuous availability of sucrose in the large seeded cultivar. Sucrose synthetase changes during mung bean seed development support the hypothesis that sucrose synthetase plays a role in starch synthesis.

The maximum rate of UDP-glucose pyrophosphorylase coincided with the maximum rate of sucrose synthetase. UDP-glucose pyrophosphorylase activity of ML-6 did not reach a level as high as those in the other two cultivars, although the activity in ML-6 at the end of the experiment was higher than those of the other two cultivars. The maximum levels of the UDP glucose pyrophosphorylase activities of CES-55 and CPI-30755A at 9–11 DAF coincided with maximum starch accumulation, suggesting that sucrose synthetase catalyses the first step in the conversion of sucrose to starch. UDP-glucose pyrophosphorylase activity fell sharply after the maximum for CES-55 and CPI-30755A suggesting that the UDP glucose pyrophosphorylase step may be the rate-limiting step in starch accumulation in the developing mung bean seed, as has been proposed to be the case in cereal grains [4].

A level of activity for UDP-glucose pyrophosphorylase, which is higher than that of ADP-glucose pyrophosphorylase in the rapid starch synthesis of the developing mung bean seed observed here, is in agreement with earlier reports [4, 6, 8]. However, the activity of ADP-glucose pyrophosphorylase was higher than the activity of UDP-glucose pyrophosphorylase at 15 DAF in ML-6 and CES-55. This may imply that UDP-glucose pyrophosphorylase comes before ADP-glucose pyrophosphorylase in the starch synthetic pathway. On the other hand, the activity of ADP-glucose pyrophosphorylase in CES-55 and ML-6 was evident at 7 DAF, at a time when UDP-glucose pyrophosphorylase activity was negligible. This may indicate that glucose-l-phosphate can also be produced through a series of reactions involving fructose [7]. This is substantiated by the fact that low activities of soluble and starch granule-bound starch synthetase, present at an early stage, may be responsible for the initiation of starch synthesis through fructose. The low activity of ADP glucose pyrophosphorylase throughout the seed development of CPI-30755A may suggest that an alternative pathway uses glucose-1-phosphate by phosphorylase [7]. On the other hand, both UDP- and ADP-glucose pyrophosphorylase showed only 0.01–0.1-times the level of

activity of sucrose synthetase and phosphorylase and could, therefore, be rate-limiting in starch synthesis.

It is noteworthy that the enzyme that might function either in starch synthesis or in glycolysis is phosphorylase. The activity of this enzyme was considerably greater than that of the other enzymes under investigation, and 10-fold higher than that necessary to support the level of starch accumulation during the course of mung bean seed development. However, maximum phosphorylase activity levels coincided with the maximum rate of starch accumulation. Low phosphorylase activities of small-seeded ML-6 at 11–15 DAF may reflect the low availability of glucose-1-phosphate because of the low activities of UDP-glucose pyrophosphorylase. Thus, it can be speculated that the activity of phosphorylase utilizing glucose-1-phosphate is operative for starch synthesis in the developing mung bean seed.

Maximum activities of soluble starch synthetase and starch granule-bound starch synthetase followed maximum activities of UDP-glucose pyrophosphorylase and ADP-glucose pyrophosphorylase in all three cultivars. This is additional evidence that starch synthesis occurs via the ADP-glucose pathway. However, the activities of both forms of starch synthetase were low at the time when starch synthesis was initiated, while there was ample phosphorylase activity. Also, the activity of soluble starch synthetase in ML-6 was lower than in the other two cultivars at the later stage of development. The data, therefore, suggest that these two forms of starch synthetase may not account for the total starch accumulation. Since the level of activity of phosphorylase was greater than that of either forms of starch synthetase at the earlier stage of development, the major portion of starch accumulation may be attributed to phosphorylase but, in the later stages, starch synthetase may be the predominant catalyst.

EXPERIMENTAL

Immature pods of mung bean cultivars ML-6, CES-55 and CPI-30755A were harvested at 5, 7, 9, 11, 13 and 15 days after flowering (DAF) from the greenhouse, where 50 plants/cultivar were grown at one plant per 20 cm i.d. pot. The developing seeds were immediately extracted from the pods in a cold room at 4° and separated into two groups. One was for carbohydrate analysis and the other for enzymatic assays.

Carbohydrate determination. To determine dry wt, the seeds were dried at 70° in a forced air drier for 72 hr. The seeds were then ground, and free sugars were extracted from the dried powder with 80% EtOH at 85° for 30 min and determined by the anthrone–H_2SO_4 method according to ref. [9]. Starch was extracted with perchloric acid from the residue of the free sugar extraction and determined by the method of ref. [10].

Preparation of enzymes. 5 g fresh developing seeds was homogenized at 0° in a blender with 10 ml 0.01 M Tris–maleate buffer (pH 7.0). The homogenate was strained and centrifuged at 30 900 *g* for 20 min at 0°. The supernatant was dialysed overnight at 4° against 0.01 M Tris–maleate buffer (pH 7.0). This soln was immediately used as a source of soluble enzymes: The pellet was washed twice with H_2O and used as a source of starch granule-bound starch synthetase.

Measurements of enzymatic activities. Sucrose synthetase (sucrose UDP-glucosyl transferase) was assayed according to ref. [11]. The reaction mixture contained 0.5 ml Tris–Pi buffer, 0.05 ml 0.4 M sucrose, 0.05 ml 5 mM 2-mercaptoethanol, 0.05 ml 5 mM UDP and 0.5 ml extract. The mixture was incubated at 37° for 10 min and then terminated by adding 1 ml of copper reagent according to Nelson's arsenomolybdate method.

ADP- and UDP-glucose pyrophosphorylase (glucose-1 phosphate adenyltransferase and glucose-l-phosphate uridyl transferase) were measured by the formation of ADP [^{14}C]glucose or UDP-[^{14}C]glucose [7]. The reaction mixture contained 0.02 ml 0.6 M Hepes buffer (pH 8.0 containing 1 mM dithiothreitol, 50 mM $MgCl_2$), 0.02 ml 12.5 mM [^{14}C]glucose-1 phosphate (300 cpm/nmol), 0.02 ml 10 mM ATP and 0.05 ml extract in a final vol. of 0.11 ml. This mixture was incubated at 37° for 20 min. The reaction was terminated by adding 0.9 ml of a soln containing 0.1 M $(NH_4)_2SO_4$, 0.01 M glucose and 0.01 M glucose-1-phosphate and the resulting mixture was then boiled for 1 min and adsorbed onto charcoal. The mixture was centrifuged at 1250 *g* for 5 min. The charcoal ppt was washed twice with H_2O mixed with 10 ml dioxan containing 0.7% PPO, 0.03% dimethyl POPOP and 10% naphthalene, and counted in a scintillation counter.

Phosphorylase assay was essentially according to ref. [12]. The reaction mixture contained 0.3 ml 2.5% (w/v) amylopectin, 0.1 ml 0.01 M glucose-1-phosphate, 0.4 ml 0.5 M Tris–maleate (pH 6.2) with 10 mM NaF, and 0.2 ml extract in a total vol. of 1.0 ml. After incubation at 30° for 30 min, 0.3 ml 5% TCA and 2.0 ml 0.1 M NaOAc were added and then the mixture was centrifuged at 1250 *g* for 15 min. Later, 2.0 ml aliquots were taken and mixed with 0.3 ml 1% (w/v) ammonium molybdate in 0.05 N H_2SO_4. Color reaction was measured after 1 hr at 650 nm.

Soluble and starch granule-bound starch synthetase (ADP-glucose starch glucosyltransferase) activities were measured by the no. of mols of [^{14}C]glucose formed from ADP-[^{14}C]glucose (0.1 μCi/μmol) with amylopectin [13]. The mixture contained 0.1 ml 0.1 M Tricine buffer (containing 10 mM EDTA, 100 mM KCl and 2.5% (w/v) amylopectin, pH 8.0), 0.02 ml 10 mM ADP-glucose and 0.08 ml extract. After incubation, at 37° for 15 min, the reaction was terminated by adding 2 ml 75% MeOH–1% KCl soln. The supernatant fraction was decanted after centrifugation at 3500 *g* for 5 min and the starch ppt was washed twice with 2 ml MeOH–KCl soln, then dissolved in 1 ml H_2O. 10 ml of scintillation soln was added before counting.

Acknowledgements—We are indebted to Messrs. Tim Griggs and Glenn Wong for valuable help with the preparation of the manuscript. Journal paper No. 71 of the Asian Vegetable Research and Development Center.

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