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Role of enzymes of sucrose-starch conversion in seed sink strength in mung bean

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Abstract

Changes in the activities of sucrose synthase (SuSy), ADP-glucose pyrophosphorylase (AGPase), UDP-glucose pyrophosphorylase (UGPase), alkaline inorganic pyrophosphatase, 3-phosphoglycerate (3-PGA) phosphatase and amylases were monitored in relation to accumulation of starch in developing pods of mung bean (*Vigna radiata* L.). With the advancement in the seed development, the contents of starch rose with a concomitant fall in the branch of inflorescence and podwall after 10 d after flowering. The activity of UDPase in all the three pod tissues remained higher than the activity of AGPase showing it to be an important enzyme controlling carbon flux. The activity of alkaline inorganic pyrophosphatase in developing seed in contrast to 3-PGA phosphatase correlated with starch accumulation rate. Activity of β-amylase increased in all the pod tissues till maturity. It appears that the cooperative action of SuSy, UGPase and AGPase controls the efficient partitioning of sucrose into ADP glucose and thereby regulate the seed sink strength of the mung bean.

Additional key words: alkaline inorganic pyrophosphatase, amylase, 3-PGA phosphatase, starch metabolism, sucrose synthase, *Vigna radiata*.

Introduction

Sucrose and starch are the major products of the carbon assimilation pathway in most plants (Pathre *et al.* 2004). The general outline of starch synthesis was thought to be fairly predictable with plastidic ADP-glucose pyrophosphorylase (AGPase) synthesizing ADP-glucose (ADPGlc), which serves as the direct precursor for starch synthesis. The presence of various enzymes of starch biosynthesis and their regulation, together with the involvement of starch degrading machinery, can be considered as defining both the quantity and quality of starch (Müller-Röber and Kossmann 1994). Recent developments in the field, including discovery of the main cytosolic isozyme of AGPase in cereal seeds (Denyer *et al.* 1996, Thorbjornsen *et al.* 1996) has called for reconsideration of the mechanisms of starch biosynthesis. Although variable mechanisms operate in different plant tissues and organs, it is often argued that the following enzymic steps are involved in the ADPGlc-synthesizing machinery located in the cytosol of starch storing cells (Pozueta-Romero *et al.* 1999):

Sucrose + UDP → UDPGlc + Fru (SuSy)
UDPGlc + PPi → G1P + UTP (UGPase)
G1P + ATP → ADPGlc + PPi (AGPase)
3-PGA → glycerate + Pi (3-PGA phosphatase)
PP → Pi (alkaline inorganic pyrophosphatase)

Kleczkowski (1994) and Denyer *et al.* (1996) put forth a hypothesis, in which sucrose synthase (SuSy) and cytosolic AGPase are coupled from various cereal seeds. Genetic and biochemical data indicate a correlation between SuSy activity and sink strength in maize endosperm and potato tuber (Zrenner *et al.* 1995, Denyer *et al.* 1996, Sharma *et al.* 1998). We have observed in mung bean and lentil seeds that SuSy activity controls the seed sink strength (Chopra *et al.* 2000, 2003). Hence, the rate of biomass accumulation and activities of AGPase, UGPase and SuSy enzymes with respect to the seed sink strength of mung bean constituted by 50 - 60 % of starch biomass were compared.

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Abbreviations: ADPGlc - ADP-glucose; AGPase - ADP-glucose pyrophosphorylase; BI - branch of inflorescence; DAF - days after flowering; G1P - glucose-1-phosphate; 3-PGA - 3-phosphoglycerate; Pi - inorganic phosphate; PW - podwall; SuSy - sucrose synthase; UGPase - UDP-glucose pyrophosphorylase; UDPGlc - UDP-glucose.
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In photosynthetic tissues, AGPase is tightly regulated by 3-PGA and inorganic phosphate (Pi), which serve as enzyme activator and inhibitor, respectively (Kleczkowski 1999). Conversely, evidence concerning regulation of 3-AGPase from non-photosynthetic tissues of plants is less clear-cut. AGPase from maize, rice and wheat endosperms and potato tuber are regulated by 3-PGA and Pi (Sivak and Preiss 1998, Gomez-Casati and Iglesias 2002). However, AGPase from pea embryos (Hylton and Smith 1992), barley endosperm (Rudi *et al*. 1997) and bean cotyledons (Weber *et al.* 1995) were found to be insensitive to regulation. To better understand the regulation of the metabolic pathway of polysaccharide accumulation, we studied the time course of accumulation of starch, and activities of AGPase, 3-PGA phosphatase and alkaline inorganic pyrophosphatase in the developing seeds of mung bean.

Though a few studies have demonstrated the presence of amylases in the developing cereal grains (Bhullar *et al.* 1985, Singh and Asthir 1988, Ziegler 1999), however, their physiological function remains unclear. Hence, it becomes essential to investigate the possible association of these enzymes with the sink capacity of legume seeds where, in comparison to cereal grains, the mechanism of starch biosynthesis has been least explored.

Materials and methods

Mung bean (*Vigna radiata* L. cv. ML 267) was raised in the field in July at 247 m above sea-level and latitude 30° 54'N following recommended agronomic practices. Fully opened flowers were tagged daily for 5 days during flowering stage, and plants of 5, 10, 15, 20 and 25 d after flowering (DAF) were collected and brought on ice to the laboratory in 5 - 10 min. The branch carrying the inflorescence was cut from the base at the point of attachment to the plant, and seeds were removed from the podwall. Each of these three fresh samples (1 - 2 g) was plunged into hot 80 % ethanol for starch estimations. Samples for the enzyme analysis were extracted immediately from the fresh tissue and their activities were assayed the same day. The temperature during extraction, isolation and centrifugation of enzyme preparations was kept at 2 - 4 °C.

For the extraction of pyrophosphorylases, fresh tissue (0.5 - 1 g) was homogenized in 3 - 4 cm^3 of 100 mM Tris-HCl buffer (pH 7.9). The homogenate was filtered through double layered cheese-cloth and centrifuged at 10 000 *g* for 15 min. The pellet was washed twice with 2 cm^3 of extraction buffer and the pooled supernatant was used immediately for assaying the activities of ADP-glucose and UDP-glucose pyrophosphorylases by coupling the product of the reaction with phospho-glucomutase and glucose-6-phosphate dehydrogenase (Turner 1969). Alkaline inorganic pyrophosphatase was extracted by homogenizing 0.5 to 1.0 g of tissue with 3 cm^3 of 0.1 M Tris-HCl buffer (pH 7.0). The homo-genate was centrifuged at 10 000 *g* for 15 min. The pellet was free of any inorganic pyrophosphatase and AGPase activity. The assay system consisted of 2.5 mM sodium pyrophosphate, 1.25 mM $MgCl_2$, 0.05 mM Tris-HCl buffer (pH 7.3) and enzyme. The contents were incubated at 37 °C for 15 min. The liberated Pi was determined (Kumar and Singh 1983). For extracting 3-phospho-glycerate phosphatase 25 mM MES-KOH (pH 6.0) buffer was used. Assay system consisting of 25 mM MES-KOH buffer (pH 6.0), 75 mM 3-PGA and enzyme was incubated at 37 °C for 15 min and Pi released was estimated (Villareal and Juliano 1977).

The extraction of α-amylase was carried out using 50 mM sodium acetate buffer (pH 5.0) containing 1 mM $CaCl_2$, and of β-amylase with 100 mM sodium acetate buffer (pH 3.6) containing 0.1 mM EDTA. In the enzyme extract used for assaying α-amylase activity, β-amylase was inactivated by heating the extract at 70 °C for 20 min. The α-amylase assay was carried out using the specific substrate, *i.e.* starch azure, following the method of Doehlert and Duke (1983). The α-amylase activity was determined from the standard curve prepared by reacting α-amylase (*Sigma*) with starch azure (Rinderknecht *et al.* 1967). β-amylase was assayed essentially according to Duffus and Rosie (1973), using starch as substrate. Sucrose synthase was extracted as described previously (Kaur *et al.* 2003) and assayed by employing the method described in detail earlier by Chopra *et al.* (2000). Conditions for linear rate with respect to time and substrate concentrations were determined for all enzymes in preliminary assays. Protein content in the enzyme extract was determined by using folin phenol reagent (Lowry *et al.* 1951).

Results

In mung bean, starch was the major constituent determining seed biomass and accumulated continuously up to maturity (Fig. 1). In branch of inflorescence (BI) and podwall (PW), starch content increased rapidly till 10 DAF and declined gradually till pod maturity. Rate of starch accumulation in mung bean seeds exhibited similar pattern as for biomass accumulation and proteins, attaining maximum value at 15 DAF.

Activity of α-amylase was maximum at 10 DAF in BI and PW and then declined (Fig. 2*A*). However, the

activity of this enzyme in the seed increased gradually from 5 to 25 DAF, though it remained lower than in the other two tissues. With the advancement of seed growth, activity of β-amylase showed an increasing trend in BI and seed and attained maximum value at maturity (Fig. 2*B*).

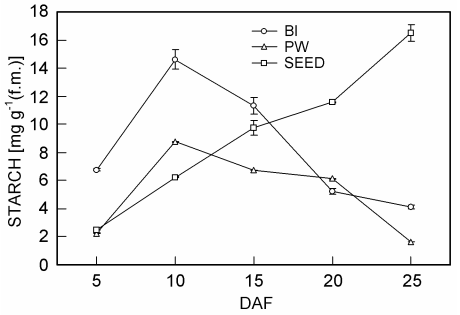

Fig. 1. Changes in starch content in branch of inflorescence (*circles*), podwall (*triangles*), and seed (*squares*) of mung bean during development. The *vertical bars* show SE of mean of three replicates. The *bars* were not shown where SE was smaller than the symbol.

Table 1. Changes in starch accumulating rate [mg seed^{-1} d^{-1}] and activities of alkaline inorganic pyrophosphatase [nmol(product formed) mg^{-1}(protein) s^{-1}] in mung bean seed during development.

DAF	Starch accumulation	Enzyme activity
5	0.09	0.02
10	0.55	0.09
15	1.45	1.01
20	1.76	0.43
25	0.10	0.18

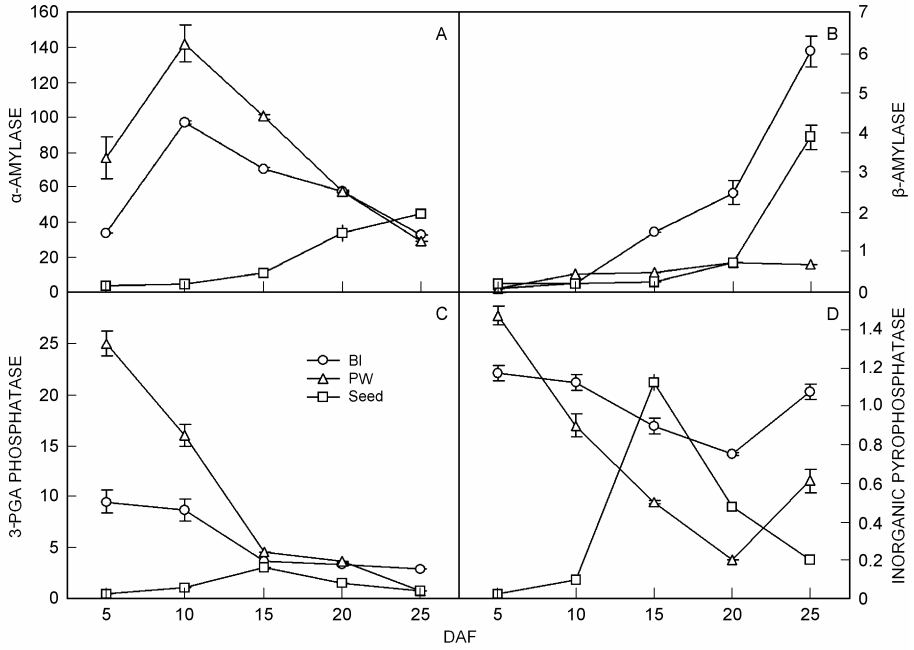

Fig. 2. Activities of starch metabolizing enzymes α-amylase (*A*), β-amylase (*B*), 3-phosphoglycerate phosphatase (*C*), alkaline inorganic pyrophosphatase (*D*) in branch of inflorescence (*circles*), podwall (*triangles*), and seed (*squares*) of mung bean during development. α-amylase activity is expressed as nmol(reducing sugar formed) mg^{-1}(protein) s^{-1}. β-amylase activity is expressed as ng(starch hydrolyzed) mg^{-1}(protein) s^{-1}. Inorganic pyrophosphatase and 3-PGA phosphatase activities are expressed as nmol(substrate hydrolyzed) mg^{-1}(protein) s^{-1}. The *vertical bars* show SE calculated from three replicates. The *bars* were not shown where SE was smaller than the symbol.

With the development of pod, activity of 3-PGA phosphatase exhibited a continuous decrease in BI and PW (Fig. 2*C*). Activity of this enzyme in the seed was observed to be at the lowest level. Activity pattern of alkaline inorganic pyrophosphatase was quite different from that of 3-PGA phosphatase. Activity of this enzyme in the seed increased sharply from 10 to 15 DAF followed by a decline till maturity (Fig. 2*D*). Compared to the seed, the enzyme activity was very high in the young podwall and declined with the development till 20 DAF and then showed a small increase at 25 DAF.

The activity of UDPglucose pyrophosphorylase in all the three pod tissues remained significantly higher than the activity of ADP-glucose pyrophosphorylase

throughout pod development (data not given). Activities of both these pyrophosphorylases attained maximum value at 15 DAF in all the three pod tissues and then declined towards maturity. Since the activities of these enzymes were predominating in the seed tissue, a comparative time course pattern was drawn for these enzymes along with sucrose synthase (cleavage) and rate of biomass accumulation in the developing mung bean seed (Fig. 3) to seek a correlation between the activities of these enzymes and seed sink strength. Like SuSy (cleavage), the activity of UGPase rose from very low values at the initial phase of the seed development to maximum values at 15 DAF (Fig. 3). The patterns of changes in the activities of all the three enzymes were almost identical, with maximum values around 15 DAF, and synchronized with the trend observed for the rate of biomass accumulation.

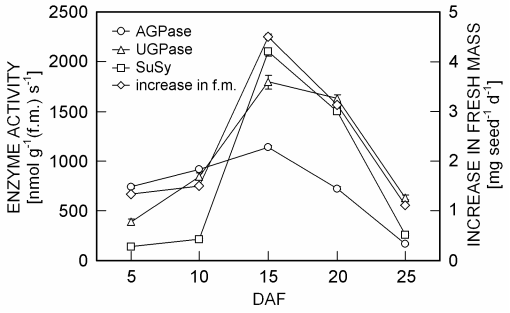

Fig. 3. Changes in fresh mass gain (*rhombs*) and enzyme activities in mung bean seeds: SuSy (*squares*), AGPase (*circles*), and UGPase (*triangles*). Fresh mass data are mean of a sample of 80 - 100 seeds collected from six randomly selected plants. The *bars* were not shown where SE was smaller than the symbol.

Discussion

Major part of starch in the mung bean seeds is accumulated between 10 - 20 DAF. A continuous deposition of starch in BI and PW upto 10 DAF with a subsequent decline reveals the transitional storage capacity of these tissues for incoming assimilates which get mobilized, at a stage of rapid accumulation in the seed (Fig. 1). In general, the podwalls of legumes not only protect the seeds therein, but are also metabolically active with respect to storing nutritional reserves for the growth of seeds (Peoples *et al*. 1985, Minamikawa *et al.* 1992). Another important finding is the presence of amylases, that, catalyze the degradation of starch in all the three tissues, *i.e*. in the branch of inflorescence, podwall and seed. Both α- and β-amylases have quite different patterns of activity throughout maturation (Fig. 2*A*,*B*). Activity of α-amylase in BI and PW rises to a maximum value and thereafter declines. The β-amylase increases steadily reaching a maximum value at maturity (Fig. 2*B*). A similar pattern of amylase activities was found in developing barley grain (Duffus and Rosie 1973). Ren *et al.* (1993a,b) identified an ethanol-soluble protein of developing soybean seeds as β-amylase, which is mainly present in cytoplasm and has many of the properties of a minor storage protein. In BI and PW, the presence of high activities of α-amylase during active pod-filling period suggest that this enzyme function in mobilization of starch that is temporarily stored in this tissue during seed development (Minamikawa *et al.* 1992). Evidence was obtained for sucrose synthase and α-amylase for having roles in mobilization of sugar reserves during growth changes in suspension cultures of French bean (Robertson *et al.* 1995). β-amylase is reported to be synthesized in the developing barley grain according to a pattern very similar to that of the major storage protein hordein (Giese and Hejgaard 1984). It is synthesized as a mature protein in the cytosol and is deposited in a 'bound' form on the surface of the starch granules when the endosperm cells lose their compartmental integrity, thus protecting it from premature attack by α-amylases (Hara-Nishimura *et al.* 1986). The production of hydrolysed products of starch by amylases for use as primers and/or for respiration during starch accumulation in cereal grain has also been suggested by some workers (Duffus and Rosie 1973, Singh and Asthir 1988, Bhatia and Singh 2000). Our data showing a steep rise of β-amylase activity at maturity indicate it to be a seed storage protein as suggested for soybean β-amylase (Ren *et al.* 1993b).

The regulation and intracellular localization of AGPase from reserve tissues are matters of controversy. The enzyme from certain storage tissues was found to be insensitive to regulation (Hylton and Smith 1992, Weber *et al.* 1995, Rudi *et al.* 1997, Doan *et al.* 1999); furthermore, it was proposed that an unregulated AGPase from cereal endosperm is located predominantly in the cytosol (Kleczkowski 1996, Thorbjornsen *et al.* 1996). Our results show a correlation between the activity of alkaline inorganic pyrophosphatase in the developing seed and starch accumulation rate (Table 1). The pattern of alkaline inorganic pyrophosphatase activity was also similar to that of AGPase (Fig. 3) indicating that removal of PPi released in the reaction catalyzed by AGPase could facilitate the conversion of sucrose to starch. However, the activity pattern of 3-PGA phosphatase (Fig. 2*C*) as such does not offer a clear understanding on the involvement of this enzyme during starch accumulation in the seed of this legume. Our results are consistent with those obtained for wheat endosperm (Kumar and Singh 1983), suspension culture cells of soybean (Gross and apRees 1986) and developing pea embryos (Hylton and Smith 1992), where AGPase is sensitive to the inhibition by orthophosphate. The enzyme from wheat endosperm was insensitive to activation by 3-PGA and sensitive to the orthophosphate regulation (Gomez-Casati and Iglesias 2002) like the mung bean enzyme. Moreover, the

possibility of the cytosolic AGPase insensitive to regulation has been proposed for cereal endosperms (Kleczkowski 1996, Thorbjornsen *et al.* 1996) with an evidence for a predominant role of the enzyme for starch synthesis.

The comparative analysis of the enzyme activities viz. SuSy, UGPase, AGPase and the rate of biomass accumulation in mung bean seed (Fig. 3) showed a correlative pattern with each other. The predominant pyrophosphorylase activity of UGPase in the mung bean seed indicate it is an important enzyme controlling carbon flux. In tissues in which sucrose degradation is metabolically important, for example, sink tissues like seeds and tubers, UGPase is believed to have a role in the synthesis of G1P (the precursor of ADPGlc synthesized by the AGPase reaction) from UDPGlc produced by SuSy (Kleczkowski 1994, Denyer *et al.* 1996). It is reported that sucrose modulates the coarse control of AGPase activities in sink tissues by affecting genetic transcription of this enzyme (Doehlert and Kuo 1990, Müller-Röber *et al.* 1990), thus indicating a close link between AGPase expression and sucrose metabolism. Our results indicate the cooperative action of sucrose synthase, UGPase and AGPase for starch biosynthesis in mung bean, thus controlling the efficient partitioning of sucrose into ADPGlc and finally controlling the seed sink strength.

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