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Phosphoenol-pyruvate-carboxylase Activity in Cotton and *Sorghum* Seeds and its Relation to Seedling Development

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Abstract. Cotton (*Gossypium hirsutum*) seeds and *Sorghum vulgare* caryopses are able to incorporate CO_2 through a PEP-carboxylating enzyme (EC 4.1.1.38). The enzyme activity is optimal at pH 8.2 and is unaffected by ATP, GDP or acetyl CoA. The partially purified cotton enzyme is stimulated by inorganic phosphate with an apparent Km of 0.3 mM. The enzymes from both cultivars are inhibited by pyrophosphate, malate, and aspartate but not by succinate. Kinetic studies for *Sorghum* and cotton seed enzymes show apparent Km values for carbonate of 5 mM and 1.2 mM and for PEP of 36 μM and 5 mM, respectively. The V_{max} values are 90 and 3.3 nmol min^{-1} mg $protein^{-1}$, respectively.

A two-fold increase in the enzyme activity from cotton seeds occurs after 2 h under laboratory germination conditions after which the activity drops sharply to 1/3 of the original activity after 5 h imbibition. No such change was observed in *Sorghum* caryopses enzyme. A correlation between PEP-carboxylase activity and seed vigor in both cultivars was demonstrated.

Key words: *Gossypium* – PEP-carboxylase – Seed vigor *Sorghum*.

Introduction

The CO_2 fixation by PEP-carboxylating enzymes in plants has been investigated intesively. Recently, the characterization of such an enzyme in maize plants was demonstrated (Uedan and Sugiyama, 1976), and the active species of "CO_2" for this enzyme were investigated (Reibach and Benedict, 1977). It has been shown that the carbons in the major components in sorghum plants derive from the carboxyl group of oxoloacetate or malate by way of the C_4 carboxylic acid pathway (Whelan et al., 1970). Although the attempts were focused on the role of the non photosynthetic CO_2 fixation in leaves, no investigations have been published so far about those activities in dry seeds.

In this paper we describe the presence of an oxaloacetate anapleorotic system, driven by a PEP-carboxy-transphosphorylase (EC 4.1.1.38) in cotton seeds and *Sorghum* caryopses.

Abbreviations: GOT = glutamicoxaloacetic-transaminase, MDH = malic dehydrogenase-$NADH_2$; RH = relative humidity

Materials and Methods

Seed Source

Commercial uniform-sized samples of cotton seeds (*Gossypium hirsutum*), cultivar Acala S.J.2 harvested in 1976; and certified uniform sized sorghum seeds cv. Hazera 610 havested in 1974, were used. The *Sorghum* caryopses were air-dried to 9.5% moisture content and kept at 4° C and 35% R.H. in well closed jars.

Seed Aging Procedure

To obtain seed samples of similar germinability but with different vigor levels, we submitted the seeds to the modified accelerated aging treatment described by Byrd and Delouche (1971). The *Sorghum* seeds imbibe moisture up to 17% at 95% R.H. and 20° C, followed by additional storage in a closed container at 30° C. After various periods (of 0–48 days, termed herein "time of aging"), seeds were air-dried down to 11% moisture and further stored at 4° C and 35% R.H. Although the germination capacity was unaffected, the seed vigor – as measured by yiled – showed an increase at 6 days of aging and then a decrease up to 48 days. At further aging the germination percentage dropped, indicating loss of viability.

Cotton seeds were treated similarly (moisture uptake up to 15% and aged up to 6 days, after which the seed viability was affected), but the seed vigor has not yet been determined.

Seed Imbibition Treatment

Seeds were placed on Whatman filter paper No. 1 in petri dishes (12 cm diameter) wetted with 6 ml water, and then incubated at 25° C.

Preparation of Seed Extract

Ten *Sorghum* seeds or ten decoated cotton seeds were ground dry with a prechiled mortar and pestle in the presence of glass beads (BDH chemicals, 40-mesh) at a ratio of 1:1 (w/w). One ml of cold 10 mM Tris-HCl buffer (pH 8.2) was added and ground again until a paste was obtained. Then 4 ml buffer for *Sorghum* and 9 ml buffer for cotton were added. The suspension was centrifuged at 4° C for 10 min at 18,000 × *g* in a refrigerated centrifuge. The supernatants were used as PEP-carboxylase enzyme source.

Partial Purification of the Cotton Enzyme

All procedures were done at 4° C. To 5 ml seed extract with a starting activity of 2.4 nmol min^{-1} mg protein, 1 g of wet DEAE-Sephadex (Cl^-) was added. The tube was shaken slightly and then centrifuged at 18,000 × *g* for 10 min. A relatively clear supernatant was obtained which contained 90% of the enzyme activity (7.5 nmol min^{-1} mg protein). The enzyme was then precipitated which 40% ammonium sulfate saturation (280 mg/ml). About 70% of the activity was recovered in the pellet fraction, which was resupended in 5 ml buffer. The activity after this step was 18 nmol min^{-1} mg protein.

PEP-Carboxylase Assay

Unless otherwise stated, the PEP-carboxylase system contained in a final volume of 1 ml: Tris-HCl buffer (pH 8.2), 50 mM; Dithioerythiol, 5 mM; $MgSO_4$, 10 mM; $Na_2^{14}CO_3$, 5 mM (10^5 cpm/μmol) for *Sorghum* and 2 mM (2×10^5/μmol) for cotton; phosphoenol pyruvate (sodium salt, Sigma), 0.1 mM for *Sorghum* and 5 mM for cotton. For acid stabilization of the product, a "trapping system" of 250 μg $NADH_2$ and 2 units of malic dehydrogenase were added. The activity was started with the addition of 0.1 ml *Sorghum* grain extract or 0.5 ml cotton seed extract (about 100 μg and 2 mg protein, respectively). After 10 min incubation at 27° C, 0.5 ml of 1 N HCl was added and the samples were boiled for 5 min in a water bath. The acid stable radioactivity was quantitated in the presence of Packard insta-Gel using a Packard Tri-Carb Scintillation Counter.

Protein Determination

Protein was quantitated in the 5% trichloroacetic acid non-soluble fraction by the method of Lowry et al. (1951).

Thin Layer Chromatography

For the examination of the carboxylation products, an assay of 0.1 ml reaction mixture was prepared with the addition of about 10^6 cpm undiluted $Na_2^{14}CO_3$ in the absence or presence of the malate or glutamic-oxoloacetic transaminase trapping system. After 20 min incubation, the acid non-stable radioactive material was liberated as described, and then the mixture was layered on Kieselgel plates and run with ethyl ether: formic acid: H_2O (7:2:1) or with butanol: acetic acid: H_2O (80:2:18) until the front reached 15 cm. An internal aspartate marker was included and detected by ninhydrin spray reagent. A separate radioactive spot of malate was run on the same plate. One-cm pices of Kieselgel from the plate were then removed and counted for radioactivity.

Results

Some characteristics of the cotton seed PEP-carboxylase are shown in Figures 1 and 2. The enzyme activity was found to be linear up to 3 mg protein/ml (not shown), and linearity is shown with time of incubation up to 10 min. The optimal pH is around 8.2 with a 50% drop at pH 7.4 or 8.6, indicating a relatively high lability to acid-base conditions.

The data shown in Table 1, namely, the inhibition by pyrophosphate (see also Fig. 3) and the ineffectiveness of CoA or GDP, indicate that the carboxylating

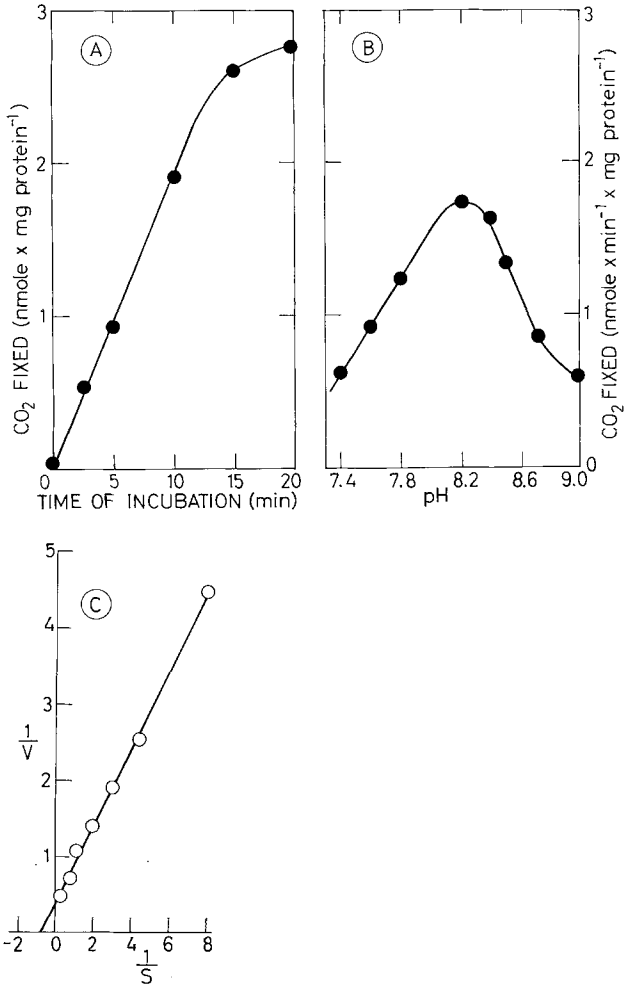

Fig. 1A–C. Characteristics of the PEP-carboxylating enzyme in cotton seed extracts. **A** Time course of CO_2 fixation. Standard assay conditions were used in the presence of the MDH trapping system. **B** Carbon dioxide fixation as function of pH. CO_2 fixation was measured at various pH values in 50 mM Tris-HCl buffers. The standard MDH assay was used. **C** Double reciprocal plot relating activity to carbonate concentration. Experimental details as in **A**

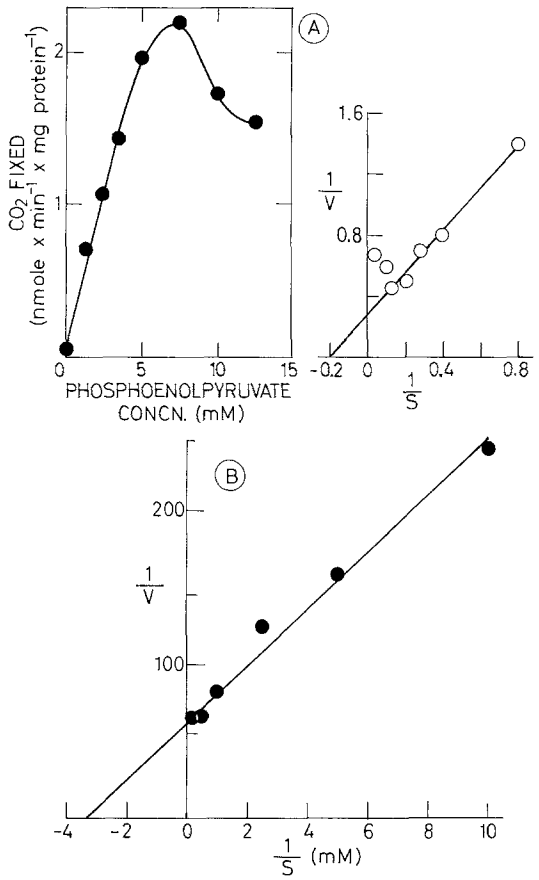

Fig. 2 A and B. The effect of PEP or inorganic phosphate concentration on CO_2 fixation by cotton seed extracts. **A** Effect of PEP concentration. Right: double reciprocal plot relating activity to PEP concentration. In routine assay mixtures, the indicated concentrations of PEP were used. **B** Double reciprocal plot relating activity to $PO_4^{=}$ concentrations. Partially purified enzyme was used. Activity was measured under standard conditions with 0.2 mg protein/assay mixture and is expressed on the ordinate in μmol min^{-1} mg $protein^{-1}$. In **A** and **B**, the MDH system was used

Table 1. The effect of various materials on PEP-carboxylating activity. In routine assay mixtures the indicated materials have been added or deleted. The malate trapping system was present except for the assay containing malate, to which the GOT trapping system was added. The rate of carboxylation was assayed as described under Material and Methods

Additions or deletions	Fixed CO_2 nmol min^{-} mg $protein^{-1}$	
	Sorghum seeds	Cotton seeds
None	52	1.7
+ATP (10 mM)	48	1.7
+ATP (10 mM) +Acetyl CoA (1 mM)	49	1.8
+Acetyl CoA (1 mM)	49	1.8
+GDP (5 mM)	51	1.8
+NADPH (10 mM)	51	1.7
+PPi (2 mM)	16	0.2
+Malate (5 mM)	8	0.3
+Aspartate (5 mM)	8	0.3
+Succinate (5 mM)	47	1.7
$-Mg^{2+}$	38	1.5
−PEP	0	0.1
−Dithioerythiol	46	1.5

enzyme present in cotton seeds is a PEP-carboxytransphosphorylase type (EC 4.1.1.38), catalyzing the reaction:

Phosphoenolpyruvate+Pi+CO_2−Oxaloacetate+PPi

This is also supported by the fact that the purified enzyme is stimulated by inorganic phosphate and exhibits a saturation curve with an apparent Km of 0.3 mM (Fig. 2). Double reciprocal plots relating the carbonate concentration or PEP concentration to the rate of carboxylation are presented. The apparent Km values are about 1.2 mM for carbonate (Fig. 1) and 5 mM for PEP (Fig. 2). The enzyme activity is inhibited by high concentrations of PEP. It should be noted that when the effect of carbonate and PEP concentrations were studied in a partially purified enzyme, no significant changes could be found in the Km value for carbonate, but for PEP it dropped to about 2 mM and no substrate inhibition could be observed.

The product of the carboxylating system was identified by two different trapping systems: 1. The malic dehydrogenase-$NADH_2$ (MDH). 2. glutamic-oxaloacetic-transaminase (GOT). The products were identified by chromatographic separations.

With MDH, 94% of the acid-stable radioactive material was found to be malate and with GOT 70% was found to be aspartate; in the absence of any external trapping system, over 90% of the radioactivity appeared at the malate spot on the chromatography plate.

The rate of enzyme activity increased two-fold during the first 2 h of seed imbibition, after which a sharp decrease occurred (Fig. 3). At 5 h imbibition the enzyme activity reached one-third of the original rate. No differences were found when the rates were compared in the presence or absence of the MDH trapping system, indicating that the change occurs in the PEP carboxylating enzyme and not in the possible trapping system.

Moreover, saturation curves for carbonate and for PEP with seed extracts from non-imbibed seeds or from seeds imbibed for 2 or 5 h showed no changes

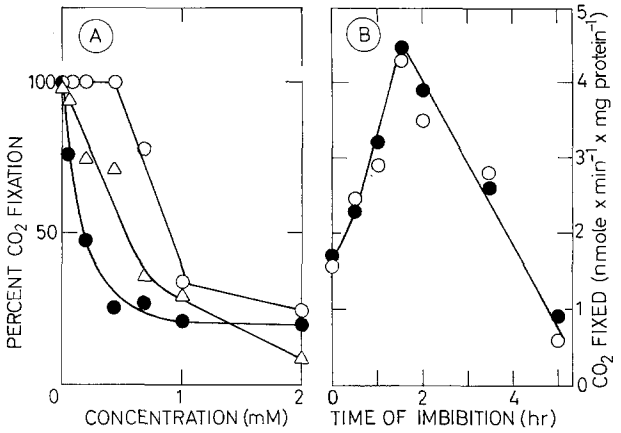

Fig. 3A and B. The effects of pyrophosphate (△), malate (●) and aspartate (○) concentrations on PEP-carboxlating enzyme from cotton seeds. **A** In standard assay mixtures the indicated concentrations of neutralized materials were added and the rate of CO_2 fixation was measured. The assays contained the MDH system except in the presence of malate – in which the GOT system was used. **B** The effect of imbibition time of cotton seeds on the PEP-carboxylase activity. The activity was measured in standard assays in the absence (●) of presence (○) of the MDH system

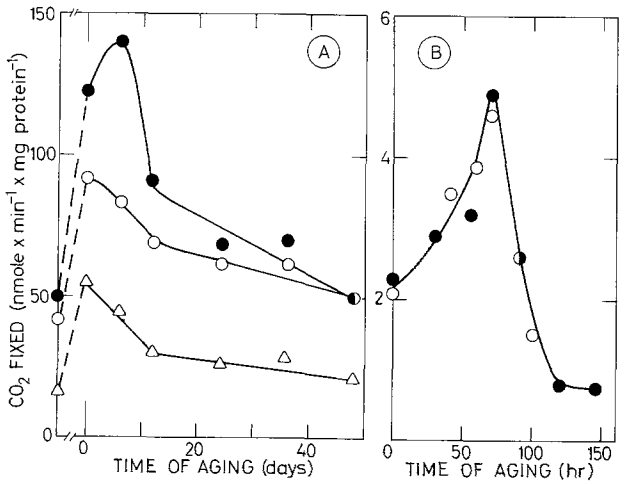

Fig. 4A and B. The correlation between accelerated aging treatments and PEP-carboxylating enzyme activity in *Sorghum* (A) and cotton (B). **A** The PEP-carboxylase activity was examined in standard assay with extracts from *Sorghum* caryopses which were incubated under aging conditions for the indicated times. The activities were measured in the absence of (△) or presence (○) of $NADH_2$ or $MDH+NADH_2$ (●). The presence of MDH alone shows a similar profile demonstrated by the triangles. **B** The PEP-carboxylating enzyme activity was examined in standard assays with extracts from cotton seeds aged in two different experiments [I – (●); II (○)] for the indicated times. The profile demonstrates the activities in the absence of the MDH trapping system. Similar activities were found in the presence of the MDH system

in the apparent Km values, but the V_{max} values were 2.4, 5.0 and 1.1 nmol min^{-1} mg $protein^{-1}$, respectively. The inhibitory effects of various concentrations of pyrophosphate, malate and aspartate are depicted in Figure 3.

PEP-Carboxylating Enzyme in Sorghum

The characteristics of the enzyme from *Sorghum* caryopses were found to be similar to those demonstrated from cotton seeds. The activity was linear with time up to 20 min and with enzyme concentration up to 500 µg protein/ml assay. The optimal pH was around pH 8.1 but the enzyme seemed to be less labile to the pH as compared to the cotton enzyme, showing 80% of the optimal activity at pH 7.2 or pH 8.5. The activity rate in the absence of an external trapping system was about 50%, as compared with the rate in the presence of the MDH system, and the acid stable product was identified to be over 95% malate.

Lineweaver-Burk plots for PEP or carbonate exhibit straight lines. The main differences were in the enzyme affinity to carbonate and PEP, and in the rate as expressed by the V_{max} value. While the Km for carbonate was about four times higher than that for cotton enzyme, the Km value for PEP (36 µM) was about 140 times lower than for the crude cotton enzyme or about 50 times lower if compared with the partially purified cotton enzyme.

Another difference was found in the rates of enzyme extracts from seeds during imbibition: no significant changes in the rate of the PEP-carboxylases were found up to 5 h imbibition; the activities fluctuated between 20 and 25 nmole min^{-1} mg $protein^{-1}$ in the absence, and between 44 and 51 nmol min^{-1} mg $protein^{-1}$ in the presence of the MDH system.

The Relation of the PEP-Carboxylating Enzyme Activity to Seed Vigor

The enzyme activity was investigated in six *Sorghum* lots with various vigor levels (as measured by their yield) and in 12 cotton seed lots which were treated by accelerated aging.

The data illustrated in Figure 4 show that the PEP-carboxylating enzyme activity increases 2–3-fold at the early time of aging in both plant seeds. In *Sorghum* seeds the activity profile followed the vigor curve, namely, an increase up to 6 days of aging, after which a sharp decrease occurs. The similar pro-

file demonstrated for cotton seed enzyme, although the differences in their vigor are not yet known, indicates that PEP-carboxylase activity in cotton seeds may also be correlated with seed vigor.

Discussion

The data presented in this paper demonstrate the presence of a PEP-carboxylase activity in cotton seeds – which is a Calvin cycle plant, and in caryoposes of *Sorghum* – a C_4 plant. The inhibitory effect of pyrophosphate, the requirement of inorganic phosphate, and the ineffectiveness of ATP, GDP and acetyl CoA, indicate that the enzyme in both cultivars is a PEP-carboxy-phosphotransphosphorylase. The inhibition by a relatively low concentration of malate suggests a possible feed-back inhibition or a product regulation. If enzyme activities in seed extracts reflect the in vivo activity, the results show that 90% of radioactive oxaloacetate is converted to malate in the absence of an external trapping system; thus, the PEP-carboxylating activity serves as an anaploerotic system providing malate to the seed cells. The increase in the enzyme activity at the early stage of germination emphasizes the importance of this enzyme at the early stages of seedling development. The possible specific role of malic dehydrogenases at the early stage of germination has already been discussed (Sato and Tasaki, 1975; Lin et al., 1976). Considering those data, in addition to the well recognized scheme that the biochemical activities in the early germination stage are predominantly related to the glycolytic activities (Brown and Wray, 1968; Burguillo and Nicolas, 1974; Moreland et al., 1974) and proceeded by an increase in the oxidative phosphorylation process, may lead to the conclusion that the PEP-carboxylating enzyme may be the malate – providing system at the early stages of seedling development. The close correlation between the enzyme activity and seed vigor in both cultivars supports the importance of the enzyme activity and its product in seedling development.

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