Photosynthesis by isolated chloroplasts of *Sorghum vulgare*

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Summary. The ambiguous location of photosynthetic carboxylases of mesophyll and bundle sheath chloroplasts of sorghum was investigated after successful homogeneous preparation. The phosphoenol pyruvate carboxylase was found as a particulate enzyme in the mesophyll cell chloroplasts and ribulose 1,5-biphosphate carboxylase in the stroma of the bundle sheath cell chloroplasts. Extensive characterization was carried out on these 2 enzymes for better understanding of the enzyme action.

Light mediated carbon assimilation by isolated chloroplasts in vitro has been well-documented[1]. This process appears to be more easily detected in chloroplasts isolated from C_3[2] plant leaves than the corresponding organelles obtained from C_4 plant species. Very few investigations are made on the latter plant group[3,4]. Probably this is due to lack of isolation techniques and difficulties in separating the 2 heteromorphic mesophyll and bundle sheath chloroplasts[5] from their respective chlorophyllous cells. The 2 chloroplasts assimilate CO_2 through phosphoenol pyruvate (PEP) and ribulose 1,5-biphosphate (RiBP) carboxylases[6], the site and location of the respective proteins within the cell types and chloroplasts remain controversial. Coombs and Baldry[7] confirmed the cytoplasmic location of PEP carboxylase in the mesophyll cell cytoplasm and RiBP carboxylase in the stroma of the chloroplasts of the same cell types. However, successful separation of individual cell types contradicted this hypothesis[8]. Our earlier investigation on the isolated cell types of sorghum leaf provide evidence of a compartmentalization of the carboxylases in different cell types[9]. In order further to strengthen this finding, the individual chloroplasts of the respective cell types were isolated and their ability of carbon assimilation in vitro was investigated. Furthermore, the site of location of the photosynthetic carboxylases were also investigated.

Materials and methods. Seedlings of *Sorghum vulgare* were grown in vermiculite free of infections under controlled growth conditions with 10-h light period at 30 °C and 45% relative humidity. Young and fully expanded 2-3-week-old plant leaves were harvested deribbed, the surface sterilized with 0.1% Na hypochlorite solution, followed by profuse rinsing in tap water, and were cut across 0.5 cm width. The 2 kinds of chlorophyllous cells, mesophyll and bundle sheath cells were isolated as previously described[9]. Class I mesophyll cell chloroplasts[10] were isolated by blending the leaf tissues in a Sorvall Omnimixer with 50% line voltage for 30 sec in 3-fold isolation buffer containing (mM) Tris-Cl 100, pH 7.3; sorbitol 300; NaCl 10; EDTA 1; DTT 5; PEG 10% (w/v) and HCO_3 10. Class II chloroplasts of bundle sheath strands in a porcelain mortar and pestle. The intactness of the chloroplasts was monitored under a phase contrast microscope after each preparation.

^{14}C-bicarbonate fixation by the isolated chloroplasts was assayed in a reaction mixture of 0.5 ml containing (μmole) Tris-Cl 100, pH 7.3; sorbitol 300; NaCl 100; $MgCl_2$ 15; DTT 3; HCO_3 15 with 2 μCi $H^{14}CO_3$ (specific activity 48.7 μCi/μmole) and chloroplast preparation containing 100–120 μg of chlorophyll. Light was supplied from a 500-W projector lamp with an intensity of 1000-foot candles at the site of reaction. After 15 min incubation at 35 °C, 0.5 ml 10% acetic acid was added to stop the reaction and to liberate the unfixed labelled bicarbonate. Aliquots were plancheted and counted in a gas flow proportional counter (Electronic Corporation of India).

Photosynthetic carboxylases were assayed as previously described[9], either by spectrophotometric or radiometric methods. Chlorophyll was estimated according to Arnon[11]. Protein was determined by the procedure described by Lowry et al[12] using BSA as standard.

Results and discussion. As a typical member of C_4 species, sorghum leaves consist of 2 kinds of chloroplastic cells, the mesophyll and bundle sheath cells[5,9], each containing its own characteristic chloroplasts, with specific photosynthetic carboxylases[6,9]. It is essential for studying in vitro photosynthesis of mesophyll chloroplasts that they be isolated as class I type[10] along with their intact outer and inner membranes. The class II types which have lost their outer membrane are easier to obtain but fail to exhibit any carbon assimilatory capabilities, even though they have the photochemical activities for which no carboxylases are required[13–15].

Successful isolation of class I mesophyll and bundle sheath cell chloroplasts was not feasible with several kinds of tropical grasses. In sorghum class I, mesophyll cells chloroplasts were obtained by direct mild mechanical blending of the leaf slices. This yielded upto 90% purity of class I type of mesophyll chloroplasts free from bundle sheath chloroplast contamination. However, class I bundle sheath chloroplasts were not easily obtainable, as they could be isolated only after the separation of bundle sheath strands. These isolated mesophyll and bundle sheath cell chloroplasts exhibited their potentialities of light-dependent ^{14}C-bicarbonate assimilation into the acid-stable photosynthetic products for a period of 15 min (figure 1) and showed 13 and 180 μmoles without any added external CO_2 acceptors (table 1). This activity was totally absent in dark. When they were supplied externally with different possible photosynthetic primary CO_2-accepting intermediary compounds,

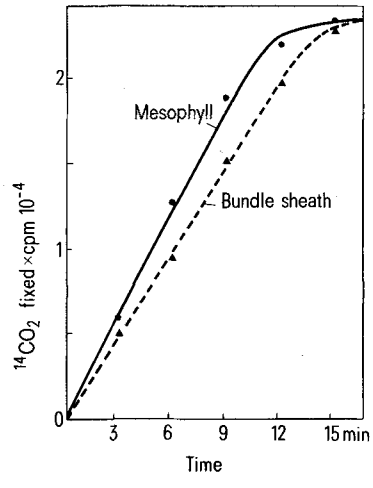

Fig. 1. Time course study on photosynthetic $^{14}CO_2$ fixation by isolated chloroplasts. The reaction was carried out in an isotonic medium adjusted to pH 7.3 at 35 °C. Light was provided from a 500-W projector lamp with a light intensity of about 10,000 lux at the site of reaction mixture.

there was an enhancement of CO_2 fixation several fold in each kind of chloroplasts. Nevertheless, the in vitro carbon assimilation of isolated chloroplasts was 2–3-fold lower than the in vivo photosynthesis[9]. In order to verify the possible mode of operation of photosynthesis in different types of chloroplasts with the existing knowledge of literature[3,6–8], the photosynthetic carboxylases of the respective cell chloroplasts were analyzed. Mesophyll cell chloroplasts contained PEP carboxylase each with an activity of 0.9 and 0.2 µmoles/mg chl/min, respectively. The presence of compartmentalization of the carboxylases in the isolated cell types has been previously reported[9,16]. The influence of the

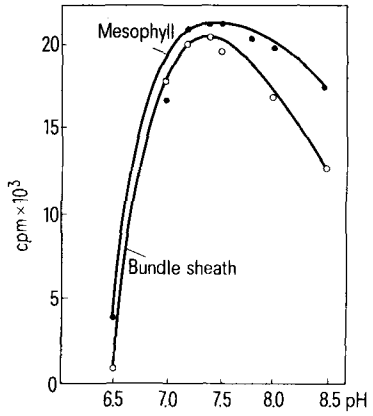

Fig. 2. Effect of pH on isolated mesophyll and bundle sheath cell chloroplasts on $^{14}CO_2$ fixation.

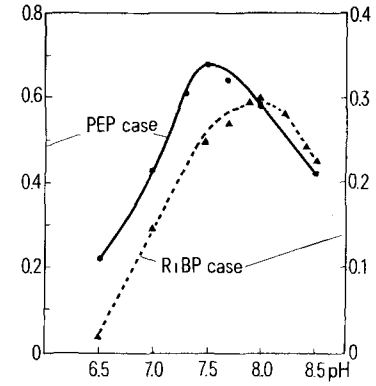

Fig. 4. Effect of pH on the photosynthetic carboxylases purified from mesophyll and bundle sheath cells.

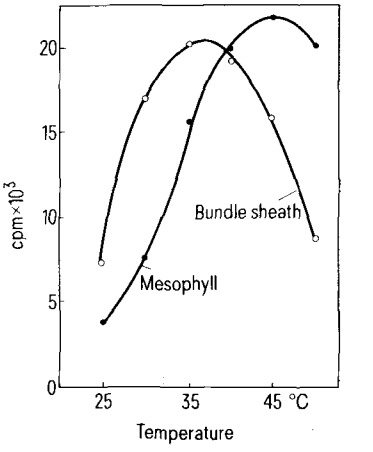

Fig. 3. Effect of temperature on isolated chloroplasts of mesophyll and bundle sheath cells on $^{14}CO_2$ fixation.

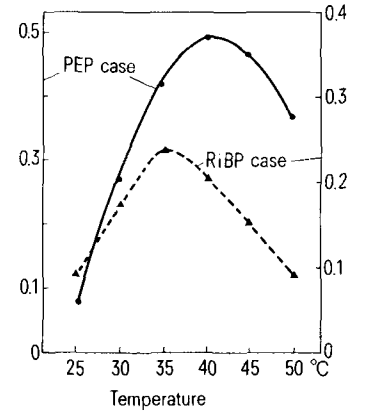

Fig. 5. Effect of temperature on PEP and RiBP carboxylases of mesophyll and bundle sheath cells, respectively.

Table 1. Effect of various photosynthetic intermediates on CO_2 fixation by the isolated mesophyll and bundle sheath cell chloroplasts under different conditions of treatment

Substrates	Mesophyll chloroplasts		Bundle sheath chloroplasts	
	Light	Dark	Light	Dark
	(µmoles $^{14}CO_2$ fixed mg^{-1} chl min^{-1})			
Nil	0.013	nil	0.18	0.03
+ Pyruvate	2.742	0.168	0.146	0.108
+ Pyruvate + ADP + Pi	2.987	0.201	0.304	0.096
+ Pyruvate + Succinate	2.874	0.304	0.374	0.083
+ PEP	4.987	1.474	0.492	0.154
+ PEP + Succinate	5.216	1.781	0.489	0.162
+ RuMP	0.092	0.043	1.941	0.174
+ RuMP + ADP + Pi	0.194	0.098	2.874	0.217
+ RuMP + Succinate	0.208	0.119	2.989	0.224
+ RiBP	0.271	0.156	3.576	0.484
+ RiBP + Succinate	0.302	0.164	3.986	0.521

^{14}C-bicarbonate fixation by the isolated chloroplasts was carried out as described under 'materials and methods'. Various photosynthetic intermediary substrates were added to the appropriate experiments at 5 mM concentration. The values are the average of 5 independent experiments.

Table 2. Characteristics of class I and II chloroplasts isolated from mesophyll cells

Treatment	Class I		Class II	
	Light	Dark	Light	Dark
	(μmoles $^{14}CO_2$ fixed mg^{-1} chl min^{-1})			
Chloroplast only	0.135	0.141	nil	nil
+ PMS	0.260	0.148	0.224	0.188
+ Pyruvate	2.824	0.134	nil	nil
+ Pyruvate + PMS	2.778	0.767	1.474	0.263
+ PEP	5.186	2.198	0.368	0.490
+ PEP + PMS	5.683	3.876	2.754	1.678
+ PEP + PMS-chloroplasts	-	1.668	-	1.104

Class I and II types were isolated as described in the text. The experimental conditions were identical to table 1. The results are the average of 5 separate experiments. PMS: Postmitochondrial supernatant; -: activities not tested. Bundle sheath cell chloroplasts were not tested on the above lines since there was no difference between the types of the chloroplasts in their activities.

ability of externally supplied intermediary photosynthetic compounds on the activity of chloroplasts further strengthens these findings. When to the mesophyll cell chloroplasts was added 5 mM pyruvate, there was about 20-fold increase in $^{14}CO_2$ fixation. Similarly the external supply of 5 mM ribulose 5-monophosphate enhanced the carbon assimilation in bundle sheath cell chloroplasts 10-fold (table 1). However, when the mesophyll and bundle sheath cell chloroplasts were supplied with the intermediary substances exchanged, there was very little enhancement, possibly due to the contaminated chloroplasts of each kind in different preparation for the assay.

Different fractions obtained from the isolated mesophyll cells revealed that the photosynthetic PEP carboxylase was found in their cytoplasmic fractions[9]. Previous observations with nonaqueous extraction techniques have shown the PEP carboxylase as a particulate enzyme in between the outer and inner membranes of the chloroplasts[17]. Evidence has been produced for the existence of chloroplastic reticulum, whereby the possible site of location of PEP carboxylase was suggested[18,19]. With these backgrounds and further experiments, the possible location of PEP carboxylase in the mesophyll cell chloroplasts has been suggested.

The mesophyll cell chloroplasts had little carbon assimilation when isolated from the separated cells, even though the whole cells showed this capacity[8,9]. In these chloroplasts, their outer membranes were damaged as observed under microscope, with lose of their carboxylase to the cytoplasm[10]. When postmitochondrial supernatant containing PEP carboxylase was added to their assay medium, there was carbon assimilatory reaction (table 2). Of the mesophyll chloroplasts when isolated directly from the leaf tissues, class I types were obtained. These organelles exhibited the light-dependent photosynthetic carbon assimilation even without added postmitochondrial supernatant (table 2). This is indicative of the presence of photosynthetic PEP carboxylase in class I chloroplasts, and its absence in class II types. This is in agreement with the earlier nonaqueous isolation of mesophyll cell chloroplasts[20], suggesting the possible particulate site of location of PEP carboxylase. However, such discrete observation did not show bundle sheath chloroplasts, irrespective of class I or II types. In these chloroplasts, the RiBP carboxylase was located within the stroma[20], and even class II types were able to show CO_2 fixation; thus further investigations were not made on them.

Incidentally, the chloroplasts were characterized for optimum pH and temperature requirements. The optimum pH was around 7.2 and 7.5 for both cells (figure 2). As a typical grass, sorghum can withstand high light intensity associated with this high temperature: hence the observed maximum CO_2 assimilation at 37 °C in mesophyll and 30 °C in bundle sheath cells (figure 3). In order to check the feasibility of the involvement of the carboxylation enzymes in these temperature-dependent reactions, the carboxylases were isolated (manuscript under preparation) and were characterised for similar pH and temperature requirements (figures 4 and 5). The PEP carboxylase exhibited a similar temperature tolerance and the pH also was high when compared to the other type of chloroplastic carboxylase, viz. RiBP carboxylase. The high temperature tolerance could be considered as one of the ecological adaptations of the mesophyll cell chloroplasts[21]. Under the above optimal conditions, the carboxylation reaction ranged between 1.6–2.1 and 0.6–1.1 μmoles/mg chl/min in mesophyll and bundle sheath cells, respectively. Further work on these 2 photosynthetic carboxylases is in progress.

1 D.M. Stokes and D.A. Walker, Biochem. J. *128*, 1147 (1972).
2 Abbreviations: BSA: bovine serum albumin; C_3: reductive pentose phosphate cycle; C_4: C_4 dicarboxylic acid cycle; DTT: dithiothreitol; PEG: polyethylene glycol; Tris:tris hydroxymethyl amino-methyl.
3 C.K.M. Rathnam and V.S.R. Das, Z. Pflanzenphysiol. *74*, 377 (1975).
4 J.A. Berry, W.J.S. Downton and E.B. Tregunna, Can. J. Bot. *48*, 777 (1970).
5 W.M. Laetsch, Sci. Prog. Oxf. *57*, 323 (1969).
6 M.D. Hatch and C.R. Slack, A. Rev. Pl. Physiol. *21*, 141 (1970).
7 J. Coombs and C.W. Baldry, Nature, New Biol. *238*, 268 (1972).
8 M.R. Gutierrez, S.C. Kanai, K. Ku and E.G. Edwards, Z. Pflanzenphysiol. *72*, 305 (1974).
9 A. Gnanam and K. Francis, Pl. Biochem. J. *3*, 11 (1976).
10 D.O. Hall, Nature, New Biol. *235*, 125 (1972).
11 D.I. Arnon, Pl. Physiol. *24*, 1 (1949).
12 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
13 K. Francis and A. Gnanam, J. Indian bot. Soc. (in press).
14 M.B. Bazzaz and Govindjee, Pl. Physiol. *52*, 257 (1973).
15 K.C. Woo, J.M. Anderson, N.K. Boardman, W.J.S. Downton, C.B. Osmond and S.W. Thorne, Proc. Nat. Acad. Sci. USA *67*, 18 (1970).
16 S.J. Kirchanski and R.B. Park, Pl. Physiol. *58*, 345 (1976).
17 T.J. Andrews, H.S. Johnson, C.R. Slack and M.D. Hatch, Phytochemistry *10*, 2005 (1971).
18 W.M. Laetsch, A. Rev. Pl. Physiol. *25*, 27 (1974).
19 C.R. Slack, M.D. Hatch and D.J. Goodchild, Biochem. J. *114*, 489 (1969).
20 R.J. Ellis, Comment Pl. Sci. *4*, 29 (1973).
21 C.C. Black, Jr, A. Rev. Pl. Physiol. *24*, 253 (1973).