BIOLOGIA PLANTARUM (PRAHA)

33 (1) : 66–74, 1991

Regulation of the Activity of Phosphoenolpyruvate Carboxylase Isolated from Germinating Maize (*Zea mays* L.) Seeds by Some Metabolites

SYLVA LEBLOVÁ, ALEXANDRA STRAKOŠOVÁ and MARTINA VOJTĚCHOVÁ

Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, CS–128 40 Praha 2, Czechoslovakia

Abstract. Phosphoenolpyruvate carboxylase (PEPC) was isolated from maize seeds which were germinated for 20 h, using a procedure which included extraction of seed homogenate with Tris-HCl or sodium phosphate buffer, precipitation of the extract with ammonium sulphate, chromatography on DEAE cellulose, and gel filtration on Sephadex G-200. Phosphate buffer was found to be less suitable than Tris-HCl buffer both for maize seed extraction and for further PEPC purification steps. The enzyme preparation obtained was electrophretically homogenous. PEPC activity was inhibited by both phosphate and malate. K_i values obtained at pH 8.1 which is the pH optimum of the reaction equelled to 42 mmol l^{-1} for phosphate and to 13 mmol l^{-1} for malate. PEPC isolated from germinating maize seeds was activated by glucose-6-phosphate, glucose-1-phosphate, ribulose-1,5-bisphosphate, fructose-1,6-bisphosphate, and fructose-2,6-bisphosphate. The authors intend to elucidate the mechanism of PEPC activation by sugars by means of the application of a number of derivatives of the sugar phosphates, among which for example 2-deoxy-2-fluoro glucosephosphate also activated PEPC. Sugar phosphates activated PEPC isolated from germinating maize seeds in this order, with increasing effect: fructose-1,6-bisphosphate, glucose-1-phosphate, glucose-6-phosphate, 2-deoxy-2-fluoro glucosephosphate, ribulose-1,5-bisphosphate, fructose-2-6-bisphosphate.

Our earlier results have shown that phosphoenolpyruvate carboxylase (PEPC), the enzyme which catalyzes the transfer of carbon dioxide to phosphoenolpyruvate which results in the formation of oxalacetate and the release of orthophosphate, is present in germinating maize seeds during the initial five days of seed germination (Ryšlavá *et al.* 1988). So far, the function of the enzyme is not well understood: Oxalacetic acid could function in germinating seeds as H^+ acceptor from NADH which is generated during the glycolysis of sugars under anaerobic conditions, or it could be utilized as a starter of the Krebs cycle, or it could supply the carbon skeleton for the synthesis of some amino acids. Other likely functions of PEPC were discussed by Latzko and Kelly (1983).

Received June 26, 1989: accepted January 29, 1990

The role of PEPC in the fixation of carbon dioxide in C–4 plants and in CAM plants was thorougly studied, for example by O'Leary (1982) and by Andreo *et al.* (1987). Phosphoenolpyruvate carboxylase present in green maize leaves has been studied for a long time in our laboratory (*e.g.* Mareš and Leblová 1980). In this paper, the possibilities of PEPC isolation from germinating maize seeds of the same cultivar which was used for the characterization of the enzyme isolated from green leaves (Stiborová and Leblová 1985) are described. Our preliminary results have shown that PEPC isolated from maize seeds is regulated by some metabolites, as for example nucleotides (Leblová and Vojtěchová 1989b), and also by sugar phosphates.

MATERIAL AND METHODS

PEPC was isolated from 20-h-germinating maize (*Zea mays* L., cv. CE 205 S) seeds. The seeds were homogenized with chilled 0.1 mol l^{-1} sodium phosphate buffer, pH 7.8, or with Tris-HCl buffer with the same molarity and pH value, to which 5 mmol l^{-1} mercaptoethanol, 5 % glycerol, 2 mmol l^{-1} EDTA, and 5 mmol l^{-1} $MgCl_2$ were added. The homogenate was centrifuged at 15 000 *g*, and the supernatant obtained precipitated with ammonium sulphate at 30 to 60 % saturation. Resuspended sediment was dialyzed for 24 h against the buffer used for homogenate preparation. PEPC was then purified by means of chromatography on an 1.8 × 40 cm DEAE-cellulose column, with linear gradient of sodium chloride from 0 to 0.25 mol l^{-1}, at a flow rate of 1 ml min^{-1}. Fractions with a volume of 3 ml were collected. Fractions with PEPC activity were combined, precipitated with ammonium sulphate at 70 % saturation, and the sediment separated by centrifugation was resuspended in 2 ml of buffer (about 5 mg of protein were obtained from 300 g of seeds). The suspension was applied onto a Sephadex G-200 column, 2.5 × 64 cm. Sample elution was carried out at a flow rate of 1 ml min^{-1}. Fractions with PEPC activity were concentrated by means of dialysis against sucrose. Thereafter they were chromatographed on a Sepharose 4B column, 2.4 × 36 cm. PEPC fractions were eluted with Tris-HCl buffer.

Different PEPC fractions obtained by means of the above isolation steps were subjected to polyacrylamide gel electrophoresis in alkaline medium according to Slustr (1972). Bands with PEPC activity were detected by incubating the gel in a mixture consisting of 0.1 mol l^{-1} Tris-HCl buffer, pH 8.0, 0.25 mmol l^{-1} phosphoenolpyruvate, 8 mmol l^{-1} violet B, 40 mmol l^{-1} $NaHCO_3$, and 30 mmol l^{-1} $MgCl_2$. Bands with PEPC activity turned black.

Abbreviations used: CAM = Crassulacean Acid Metabolism; DEAE-cellulose = diethylaminoethyl cellulose; EDTA = ethylenediaminotetraacetic acid; NAD = nicotinamide adenine dinucleotide; NADH = reduced form of nicotinamide adenine dinucleotide; Tris = tris(hydroxymethyl)aminomethane; PEPC = phosphoenolpyruvate carboxylase.

TABLE 1

The isolation of PEPC from germinating maize seeds. The values presented in the table correspond to the preparation obtained from 100 g of seeds. A – sodium phosphate buffer; B – Tris-HCl buffer.

Purification step	Protein content [mg]		PEPC activity [nmol s^{-1}]		PEPC specific activity [nmol s^{-1}]		Degree of purification mg^{-1}	
	A	B	A	B	A	B	A	B
Crude extract	1800	1571						
Sulphate fraction	2017	867	78.3	51.7	0.0388	0.0596	1	1
Fraction after DEAE cellulose Chromatography	167	92.3	52.6	42.5	0.3149	0.4604	8.1	7.7
Fraction after gel filtration on Sephadex G–200	56.7	27.6	23.9	18.3	0.4215	0.6630	10.9	11.1

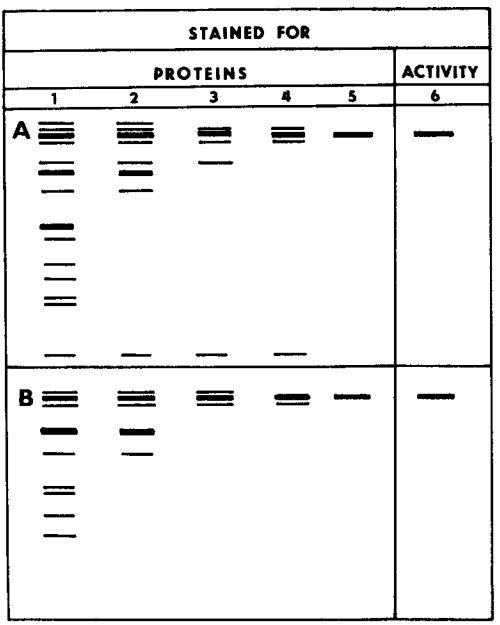

Fig. 1. Electrophoreograms of PEPC preparations obtained in different steps of PEPC isolation from germinating maize seeds. A – with sodium phosphate buffer; B – with Tris-HCl buffer; detection of protein 1–5, detection of PEPC activity (6). 1. Crude extract from germinating seeds; 2. ammonium sulphate fraction; 3) combined fractions with PEPC activity after the chromatography on DEAE-cellulose; 4) combined fractions with PEPC activity following gel filtration on Sephadex G–200; 5) combined fractions with PEPC activity following the chromatography on Sepharose 4B.

PEPC activity was determined by using two methods: according to absorbance increase due to increasing oxalacetic acid concentration at 280 nm, or according to absorbance decrease at 340 nm due to the oxidation of NADH in a reaction coupled with malate dehydrogenase (Leblová *et al.* 1989a).

TABLE 2

K_i values and the type of inhibition of PEPC isolated from maize seeds caused by phosphate ions

pH	$K_{i(PEP)}$ [nmol l^{-1}]	Type of inhibition	$K_{i(Mg^{2+})}$ [nmol l^{-1}]	Type of inhibition
6.0	42	C	39	C
7.0	10	C	25	C

C = competitive

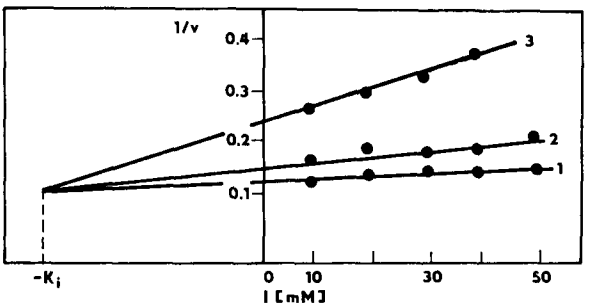

Fig. 2. The effect of phosphate ions on the velocity of the reaction catalysed by PEPC. Abscissa: phosphate concentration [mmol l^{-1}]; ordinate: 1/v [$mmol^{-1}$ min]. *1*–5.0 mmol l^{-1} Mg^{2+} *2*–2.0[mmol l^{-1}] Mg^{2+} *3*–0.5.

Proteins were determined according to Lowry *et al.* (1951) using bovine albumin as a standard.

RESULTS AND DISCUSSION

PEPC can be isolated from germinating maize seeds by extracting it either with sodium phosphate or with Tris-HCl buffer, as documented in Table 1. The extraction with phosphate buffer resulted in a higher amount of non-PEPC proteins in the extract obtained. The degree of purification is not accurate, because PEPC activity cannot be reliably determined spectrophotometrically in raw extracts of maize seeds.

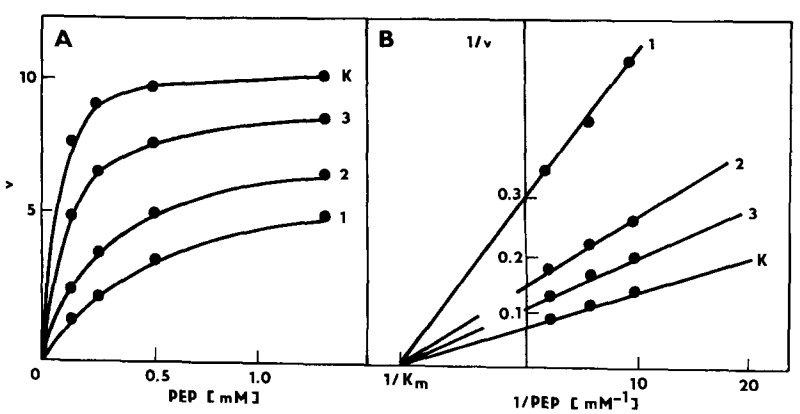

Fig. 3A. The dependence of the velocity of the reaction catalysed by PEPC on the concentration of malate as an inhibitor. Abscissa: PEP concentration [mmol l^{-1}]; ordinate: reaction velocity v mmol min^{-1} *1*–1.0 mmol l^{-1} malate *2*–0.5 mmol l^{-1} malate *3*–0.25 mmol l^{-1} malate.

Fig. 3B. The dependence of reciprocal initial PEPC velocity on reciprocal PEP concentration in the presence of malate. Abscissa: 1/PEP [mmol l^{-1}]; ordinate 1/v [mmol min]. *1*–1.0 mmol l^{-1} malate, *2*–0.05 mmol l^{-1} malate, *3*–0.25 mmol l^{-1} malate.

TABLE 3

The effect of 0.05 mmol l^{-1} concentration of sugar derivatives on the activity of PEPC isolated from germinating maize seeds.

PEP concentration [mmol l^{-1}]	Activator										
	none	glc-6P		glc-1P		fru-2, 6-BP		rul-1, 5-BP		2-deoxy-2-fluoro glc-6P	
	V_0	V	V/V_0	V	V/V_0	V	V/V_0	V	V/V_0	V	V/V_0
0.10	50	65	1.3	75	1.5	133	2.6	122	2.4	94	1.9
0.25	65	90	1.4	85	1.3			123	1.9	106	1.6
0.90	100	120	1.2	100	1.0			106	1.1	105	1.1
0.75	100	120	1.2	100	1.0			108	1.1	105	1.1
1.00	100	135	1.4	100	1.0			108	1.1	106	1.1

The values presented in the table represent % of activity. V_0 = PEPC activity without the modulator, V = PEPC activity with the modulator, as determined at the pH optimum 8.0.

PEPC obtained by the procedure described in Table 1 can be used for kinetic studies. The inclusion of chromatograpy on Sepharose 4B results in a homogenous PEPC preparation, as documented in Fig. 1.

Sodium phosphate buffer inhibits PEPC in a molarity range from 10 to 50 mmol l . Phosphate functions as a competitive inhibitor both against the substrate, phosphoenolpyruvate and against the cofactor, that is Mg^{2+} ions. The values of the inhibition constants are dependent on pH values of the assay mixture medium (Table 2, Fig. 2).

Malate at concentrations from 0.25 to 1.0 mmol l^{-1} acts as a non-competitive inhibitor of PEPC with respect to phosphoenolpyruvate (Figs. 3A, 3B). The magnitude of the inhibition constant is, similarly as in case of phosphate, dependent on assay mixture pH: it equals 0.87 mmol l^{-1} at pH 7.0, whereas at pH 8.0 which is the pH optimum of the reaction catalyzed by PEPC, it amounts to 13.0 mmol l^{-1}. Similar results were recorded with PEPC isolated from green leaves of maize,

TABLE 4

The effect of phosphate derivatives of sugars on the activity of PEPC, isolated from maize seeds, as determined at its pH optimum of 8.1; the values presented in the table represent % of activity, with 100 % being PEPC activity without modulators and with the saturation concentration of the substrate PEP

PEP concentration [mmol l^{-1}]	Ribulose-1,5-bisphosphate concentration [mmol l^{-1}]					
	0	0.5	1.0	1.5	2.0	3.0
0.10	50	122	133	144	177	188
0.25	65	123	130	131	130	132
0.50	100	116	133	133	128	127
1.00	100	108	108	108	108	123
	2-Deoxy-2-fluoro glucosephosphate concentration [mmol l^{-1}]					
	0	0.5	1.0	1.5	2.0	3.0
0.10	4	42	44	50	55	53
0.25	67	78	72	78	78	78
0.50	83	89	100	100	100	100
0.75	92	105	105	100	98	111
1.00	100	105	105	111	114	111
	Fructose-2,6-bisphosphate concentration [mmol l^{-1}]					
	0	0.5	1.0	1.5	2.0	3.0
0.10	50	66	72	72	74	76
1.00	100		133		133	144

sorghum, and millet. Inhibition constants determined at pH optima were in the range from 4.5 to 7.0 mmol l^{-1} according to the plant species.

PEPC isolated from green leaves was markedly activated with glucose-6-phosphate (Mukerji 1977). In *Pennisetum purpureum*, glucose-6-phosphate activated PEPC at high concentrations, whereas it inhibited it at low concentrations (Coombs *et al.* 1973). Our earlier experiments have shown (Stiborová and Leblová 1985) that the activation of the enzyme isolated from green maize leaves is dependent on the concentration of glucose-6-phosphate (it increases in the range from 0 to 30 mmol l^{-1}) and on pH of the medium (it is higher at pH 7.0 than at pH 8.0 which is the pH optimum of PEPC), but independent of the concentration of magnesium ions. PEPC activity increased at its pH optimum in the presence of 30 mmol l^{-1} of glucose-6-phosphate by 86 % when compared with the control, whereas by 167 % at pH 7.0. The activity of PEPC isolated from maize seeds is enhanced by sugar phosphates (Tables 3 and 4). The activation is clear-cut especially at low substrate (phosphoenolpyruvate) concentrations. A non-physiological derivative of glucose, 2-deoxy-2-fluoro glucose-6-phosphate, also activates phosphoenolpyruvate carboxylase. The sugar derivatives employed are arranged according to the magnitude of their activation coefficients in Table 5. We shall attempt to obtain a larger number of sugar derivatives or of their phosphate esters and to employ them for the elucidation of the interaction of the studied activators with PEPC molecule.

TABLE 5

Activation constants of some sugar derivatives for PEPC, isolated from germinating maize seeds, as determined in Tris-HCl buffer, pH 6.1

Modulator	K_A [mmol l^{-1}]
Fructose-1,6-bisphosphate	33.1
Glucose-1-phosphate	24.0
Glucose-6- phosphate	24.0
2-Deoxy-fluoro glucosephosphate	10.0
Ribulose-1,5-bisphosphate	2.2
Fructose-2,6-bisphosphate	0.29

We assume that the study of PEPC isolated from germinating maize seeds will contribute to the understanding and specification of similarity to, or dissimilarity from PEPC present in green leaves, leaves grown in the dark, and roots, and to the understanding of the function of phosphoenolpyruvate carboxylase in the process of seed germination. PEPC isolated from maize seeds resembles in the extent of its activation with glucose-6-phosphate more the enzyme isolated from C-3 plants than the enzyme which is present in green leaves of C-4 plants which is regulated to a grater extent (Ting and Osmond 1973).

RERERENCES

Andreo, C. S., Gonzales, D. H., Iglesias, A. A.: Higher plant phosphoenolpyruvate carboxylase. Structure and regulation. – FEBS Lett. **213** : 1–8, 1987.

Coombs, J., Baldry, C. W., Bucke, C.: The C-4 pathway in *Pennisetum purpureum*. The allosteric nature of PEP carboxylase. – Planta **110** : 95–107, 1973.

Latzko, E., Kelly, G. J.: The many-faceted function of phosphoenolpyruvate carboxylase in C-3 plants. – Physiol. vég. **21** : 805–815, 1983.

Leblová, S., Strakošová, A., Vojtěchová, M.: Isolation and parcial characterisation of phosphoenolpyruvate carboxylase from germinating seeds of maize (*Zea mays*). – Biológia (Bratislava), **12** : 1161–1169, 1989.

Leblová S., Vojtěchová, M.: The effect of some adenine and guanine nucleotides on phosphoenolpuryvate carboxylase isolated from germinating seeds of maize (*Zea mays*). – Biológia (Bratislava), **12** : 1171–1176, 1989.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: Protein measurement with the Folin phenol reagent. – J. biol. Chem. **193** : 265–275, 1951.

Mareš, J., Leblová, S.: Phosphoenolpyruvate carboxylase from leaves of maize, sorghum and millet. – Photosynthetica **14** : 25–31, 1980.

Mukerji, S. K.: Corn leaf phosphoenolpyruvate carboxylase: Inhibition or $^{14}CO_2$ fixation by SO_3^{2-} and activation by glucose-6-phosphate. – Arch. Biochem. Biophys. **182** : 360–365, 1977.

O'Leary, M. H.: Phosphoenolpyruvate carboxylase: an enzymologist's view. – Annu. Rev. Plant Physiol. **33** : 297–315, 1982.

Ryšlavá, H., Strakošová, A., Leblová, S.: Oxygen stress in plants: Is phosphoenolpyruvate carboxylase involved in the metabolism processes during natural anaerobiosis – In: Abstracts of 14th International Congress of Biochemistry. P. 199. Praha 1988.

Slustr, L.: Preparative acrylamide gel electrophoresis: continuous and disc techniques. – In: Colowick, S., P., Kaplan, N. O. (ed.): Methods in Enzymology. Vol. 22. Pp. 412–433. Academic Press, New York–London 1972.

Stiborová, M., Leblová, S.: Activation of maize (*Zea mays* L.) phosphoenolpyruvate carboxylase by glucose-6-phosphate and glycine: regulation of activation by pH. – Photosynthetica **19** : 177–182, 1985.

Ting, I. P., Osmond, C. B.: Photosynthetic phosphoenolpyruvate carboxylase: characteristics of alloenzymes from leaves of C–3 and C–4 plant. – Plant Physiol. **51** : 439–447, 1973.