Purification and stability during storage of phoshoenolpyruvate carboxylase from leaves of *Amaranthus hypochondriacus*, a NAD-ME type C_4 plant

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Abstract

A traditional method is reported for purification of phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) from leaves of *Amaranthus hypochondriacus* L. with a high yield of 50 %, 135-fold purification, and specific activity of 900 mmol kg^{-1}(protein) s^{-1}. PEPC was purified from light-adapted leaves of *A. hypochondriacus*, involving 40-60 % ammonium sulphate fractionation, followed by chromatography on columns of DEAE-*Sepharose*, hydroxylapatite (HAP), and *Seralose 6-B*. The enzyme appeared as a single band on 10 % SDS-PAGE, with a molecular mass of about 100 kDa. Kinetic studies with purified enzyme confirmed the PEPC to be the light-form of the enzyme. Glycerol generally increased the stability of PEPC. The stability and storage of the purified enzyme was studied at temperatures of 4 °C, -20 °C, and liquid nitrogen. PEPC maintained its activity for up to 3 months upon storage with 50 % (v/v) glycerol in liquid nitrogen.

Additional key words: DEAE-*Sepharose*; glucose-6-phosphate; glycerol; hydroxylapatite; L-malate; phosphate; phosphoenolpyruvate; *Seralose-6B*.

Introduction

Cytosolic phosho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) plays a cardinal role in the fixation of atmospheric CO_2 during C_4 photosynthesis and CAM (Rajagopalan *et al.* 1994, Chollet *et al.* 1996, Vidal and Chollet 1997). The expression of PEPC activity is controlled at both the transcriptional and posttranslational levels (Chollet *et al.* 1996). Posttranslational controls include modulation of enzyme activity *via* allosteric effects and by reversible phosphorylation: PEPC is activated by glucose-6-phosphate (G-6-P) and (only in C_4 monocots) by glycine; it is inhibited by L-malate (Lepiniec *et al.* 1994). Therefore, to study the regulatory phosphorylation of PEPC both *in vivo* and *in vitro*, it is often necessary to purify the enzyme by a simple and rapid method.

A vast amount of literature is available on purification and kinetic properties of the plant enzyme (for reviews see Toh *et al.* 1994, Chollet *et al.* 1996). However, very few studies have been done on storage of the purified enzyme and maintenance of its stability. The properties of the enzyme can vary depending on the assay pH, presence and absence of glycerol, and storage conditions. So far there are no reports regarding purification and stability of PEPC from *A. hypochondriacus*. Therefore, a study on the purification and the kinetic properties of the purified enzyme stored at different temperatures, in the presence and absence of 50 % (v/v) glycerol, was done.

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Abbreviations: G-6-P, glucose-6-phosphate; HAP, hydroxylapatite; NAD-ME, NAD-malic enzyme; PAGE, polyacrylamide gel electrophoresis; PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate carboxylase; SDS, sodium dodecyl sulphate.

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Materials and methods

Plants of *A. hypochondriacus* L. (cv. AG-67) were raised from seeds, supplied from the Cytogenetics Division, National Botanical Research Institute, Lucknow. The plants were grown in soil supplemented with farm-yard manure, in 25-cm diameter earthen pots, and kept outdoors in the field (approximate photoperiod of 12 h and temperature of 30-40/25-30 °C day/night). The upper fully expanded leaves of 4-6 week old plants were harvested, about 2-3 h after sunrise.

PEPC activity was assayed by coupling to NAD-malic dehydrogenase. Enzyme activity was determined at 30 °C by monitoring NADH oxidation at 340 nm in a dual beam UV-Vis spectrophotometer *Shimadzu UV-160A* (Japan). The reaction mixture (1 cm^3) contained 50 mM Hepes-KOH (pH 7.3), 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 units of NAD-malic dehydrogenase, 0.2 mM NADH, and leaf extract equivalent to 1 μg protein. The extract or purified enzyme was incubated in the assay medium for 30 s to initiate PEPC. This minimizes the dissociation of tetramer to dimer upon dilution. The reaction was started by addition of 50 cm^3 of 50 mM phosho*enol*pyruvate (stock solution of PEP prepared in 50 mM Hepes-KOH, pH 7.3). The reaction was linear for at least 8 min with crude extracts and for 5 min with purified enzyme.

The maximum velocity of the enzyme (V_{max}) and the K_m for PEP were examined in the presence or absence of glycerol. The enzyme was first incubated for 30 s in the assay medium and the reaction was started by addition of PEP (0.5 to 5 mM final concentration). K_m was calculated from the Lineweaver-Burk plots.

Malate sensitivity was determined by inclusion of 0 to 5 mM L-malate. K_i values were calculated from a linear inhibition-equation using a computer program developed by Brooks (1992). The activation of PEPC by G-6-P was also studied in a similar manner as described above, except that different concentrations of G-6-P (0 to 5 mM) were added instead of L-malate in the assay medium. K_A (G-6-P) values were calculated by an activator equation, using the computer program (Brooks 1992). The stock solutions of L-malate and G-6-P were prepared in 50 mM Hepes-KOH, pH 7.3.

The purification of PEPC is an improved method for that of *A. viridis* (Iglesias *et al.* 1986). Leaves (40 g) of *A. hypochondriacus* were harvested, washed, cut into small pieces, and suspended in 200 cm^3 of buffer containing 100 mM phosphate buffer, pH 7.2, 25 % (v/v) glycerol, 5 mM DTT, 5 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM EDTA, 2 mM PMSF, and 10 mM 2-mercapto-ethanol. Solid polyvinyl-pyrrolidone (0.5 g per 1 g) was added to the medium. The leaves were then homogenised using a Waring blender (1.5 min; maximum speed). The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 15 000×*g* for 10 min. The above procedure was performed at 4 °C and the subsequent steps were carried out at room temperature of about 20-22 °C since the enzyme loses some of its activity when prepared at 4 °C (Zervoudakis *et al.* 1998).

The supernatant (300 cm^3) was brought to 40 % saturation with saturated ammonium sulphate solution. The suspension was stirred slowly for 30 min and then centrifuged at 15 000×*g* for 15 min. The precipitate was discarded, the supernatant was brought to 60 % saturation by further addition of saturated ammonium sulphate solution, and the precipitate was collected by centrifugation at 15 000×*g* for 30 min. The above precipitate was suspended in 5 cm^3 of 200 mM potassium phosphate buffer (pH 7.2) plus 10 % (v/v) glycerol. The suspension could be stored overnight at 4 °C without loss of enzyme activity. The solution was dialyzed against 20 mM potassium phosphate buffer (pH 7.2) and 10 % (v/v) glycerol and then loaded onto a DEAE-*Sepharose CL-6B* (*Pharmacia*) column (1×7 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.2) and 10 % (v/v) glycerol. The column was washed with the same buffer at a flow rate of 8.3 mm^3 s^{-1} until A_{280nm} returned to baseline. A linear gradient of 20 to 200 mM potassium buffer (pH 7.2) containing 10 % (v/v) glycerol was used to elute PEPC. The active fractions were pooled (20 cm^3) and the enzyme was precipitated with 60 % (v/v) saturated ammonium sulphate solution.

The hydroxylapatite (HAP) column was prepared in the laboratory as described by Oishi (1971). The precipitate from the above step was dissolved in 200 mM phosphate buffer, pH 7.2, containing 10 % (v/v) glycerol and dialyzed against 20 mM phosphate buffer (pH 7.2) with 10 % (v/v) glycerol. The dialyzed sample was applied to a 1×7 cm HAP column. The dialyzed eluate was applied slowly and the eluate which passed out the column was recycled (5 to 6 times). This ensures complete binding of the enzyme to the column and the removal of non-specific proteins from the column. PEPC was eluted with a linear gradient of 20-200 mM phosphate buffer (pH 7.2) plus 10 % (v/v) glycerol. The active fractions were pooled and then precipitated with saturated ammonium sulphate solution (60 %, v/v).

The precipitate was dissolved in 20 mM potassium phosphate buffer (1.5 to 2 cm^3) containing 10 % (v/v) glycerol and applied onto a column (1×25 cm) of *Seralose 6-B* (its analytical grade is a beaded form of agarose and is similar to *Sepharose*) equilibrated with 20 mM potassium phosphate buffer with 10 % (v/v) glycerol. PEPC was eluted with a linear gradient of

20-200 mM of the same phosphate buffer as a single peak at a flow rate of 4.17 mm^3 s^{-1}. The fractions containing high activity were pooled and concentrated with solid PEG 20 000. The concentrated, purified PEPC was stored in multiple aliquots with 50 % (v/v) glycerol in liquid nitrogen.

SDS-PAGE was performed according to Laemmli (1970). Protein bands were visualized either by Coomassie Brilliant Blue or by silver staining (Blum *et al.* 1987). A set of molecular mass markers (29 to 116 kDa) was used as standard for assessing molecular mass of proteins on SDS gels.

Native gels were run as described by Davis (1964). Twenty µg of purified PEPC was loaded in each well. A two-dimensional electrophoresis system (native/SDS-PAGE) was performed to confirm the electrophoretic behaviour and subunit composition of PEPC (Vance and Stade 1984). A set of molecular mass standards (29-205 kDa) were included during SDS-PAGE in the second dimension. Activity staining for PEPC was carried out at 30 °C as described by Nimmo and Nimmo (1982). Controls were run without PEP in the staining mixture.

The stability of the enzyme was studied by storing the enzyme at different temperatures (room temperature, 4 °C, -20 °C, liquid nitrogen) in the presence or absence of 50 % (v/v) glycerol. The properties of the enzyme were examined after its storage for either 24 h or up to 3 months.

Total protein concentration was determined using the Folin-phenol reagent (Lowry *et al.* 1951) with bovine serum albumin as standard.

Results

PEPC from *A. hypochondriacus* was purified to homogeneity by 40-60 % ammonium sulphate fractionation, followed by DEAE-*Sepharose*, HAP, and finally *Seralose 6-B* column chromatography. On passage through the DEAE-*Sepharose* column, PEPC was eluted as a broad peak at around 70-80 mM P_i (Fig. 1*A*) with a maximal specific activity of 253 mmol s^{-1} kg^{-1}(protein). On HAP, PEPC eluted as a single peak around 60-80 mM P_i (Fig. 1*B*) and the enzyme had a high specific activity of 867 mmol s^{-1} kg^{-1}(protein). The purified enzyme (after elution from *Seralose 6-B*) had a specific activity of 900 mmol s^{-1} kg^{-1}(protein) and was eluted as a single peak (Fig. 1*C*). This is one of the highest specific activities reported for PEPC from C_4 plants with a high yield of about 50 % (Table 1). The enzyme appeared as a single band on 10 % SDS-PAGE with a subunit MM of about 100 kDa (Fig. 2).

The enzyme appeared as two distinct bands following non-denaturing electrophoresis when stained for PEPC activity (Fig. 3). In a two-dimensional electrophoresis these bands merged as a single major broad band following non-denaturing/SDS-PAGE (values not shown) indicating their identical subunits.

A kinetic examination of PEPC from *A. hypochondriacus* revealed a V_{max} of 900 mmol s^{-1} kg^{-1}(protein) and K_m for PEP of 0.4 mM, at pH 7.3 (Table 2). The enzyme was activated by G-6-P with a K_A of 0.3 mM and inhibited by L-malate with a K_i of 0.5 mM.

At ambient room temperature in the absence of glycerol, the purified PEPC lost almost completely its activity within 24 h (Table 3). The enzyme in the absence of glycerol and after 24 h of storage retained only 16 and 30 % of its initial activity at 4 and -20 °C, respectively, whereas in liquid nitrogen the enzyme retained 50 % of its activity. However, on the addition of 50 % (v/v) glycerol, the enzyme retained 27 % of its initial activity at room temperature, and maintained >77 % of its initial activity at 4 °C, -20 °C, and in liquid nitrogen (Table 3). During periods of up to 3 months, the enzyme largely retained its full activity only when stored

Table 1. Purification of PEPC from leaves of *Amaranthus hypochondriacus*.

Step	Total activity [µmol s^{-1}]	Total protein [mg]	Specific activity [mmol s^{-1} kg^{-1}(protein)]	Purification [fold]	Yield [%]
Crude extract	2.70	410.0	6.7	-	100
40-60 % $(NH_4)_2SO_4$	2.53	117.0	21.7	3	93
DEAE-*Sepharose*	2.27	9.0	253.3	38	83
Hydroxylapatite	1.47	1.7	866.7	130	54
Seralose 6-B	1.35	1.5	900.0	135	49

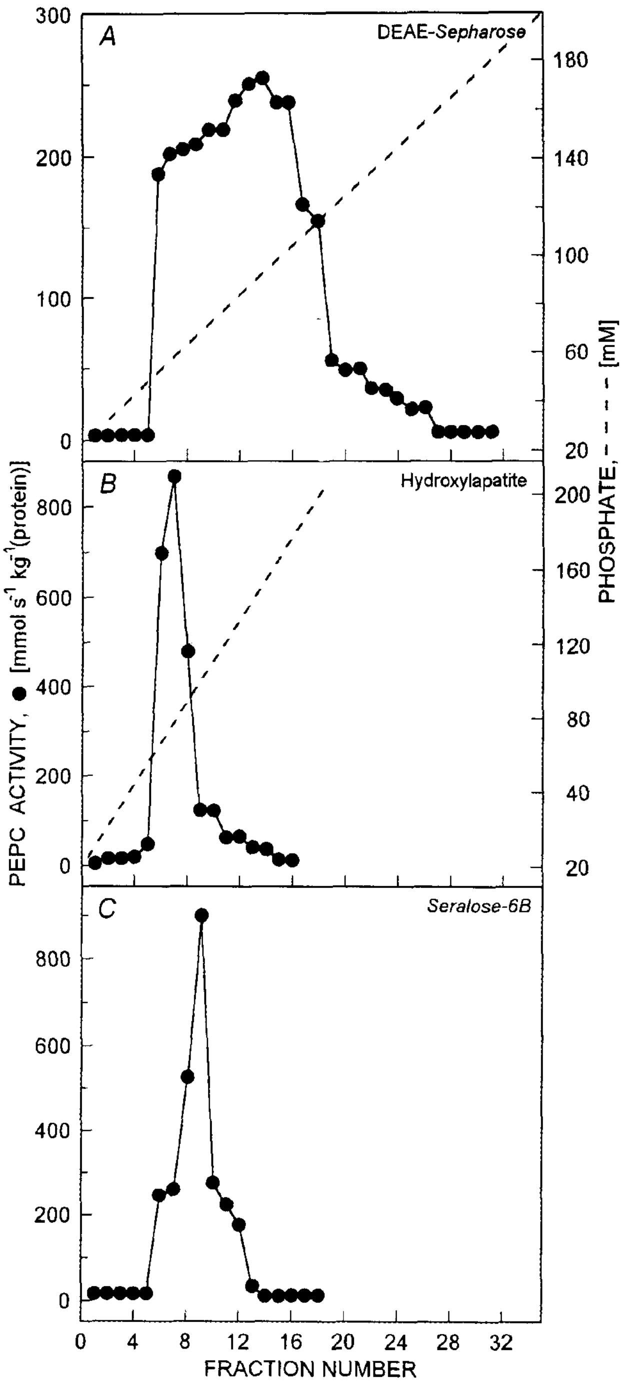

Fig. 1. The elution pattern of PEPC activity from the successive three columns: DEAE-*Sepharose* (*A*), hydroxylapatite (*B*), and *Seralose 6-B* (*C*). Further details are described in the text.

in liquid nitrogen in the presence of 50 % (v/v) glycerol (Fig. 4). PEPC showed a marked decrease in activity after 2 months at either -20 or 4 °C. This decrease in activity was associated with a decrease in malate sensitivity.

The kinetics of purified PEPC was studied after 24 h of storage. The V_{max} of the enzyme was maintained only at liquid nitrogen temperature in the presence of 50 % (v/v) glycerol (Table 4). The affinity of PEPC for PEP remained unaltered when stored in liquid nitrogen, while the affinity increased at even -20 °C (Table 4). K_A for G-6-P increased at 4 and -20 °C in comparison to PEPC stored in liquid nitrogen. The high specific activities of the enzyme, malate sensitivity, response to G-6-P, and affinity towards PEP were all maintained (close to the values of freshly purified enzyme) only in the presence of glycerol. The malate sensitivity and specific activity of the enzyme were stable when stored in liquid nitrogen with 50 % (v/v) glycerol for 3-4 months.

Table 2. Kinetic characteristics of PEPC purified from leaves of *A. hypochondriacus*. Means ± SE from five independent experiments.

Parameter	Value
V_{max} [mmol s^{-1} kg^{-1}(protein)]	900.00 ± 1.00
K_m PEP [mM]	0.40 ± 0.02
K_i L-malate [mM]	0.50 ± 0.01
K_A glucose-6-P [mM]	0.30 ± 0.06

Table 3. The stability of purified PEPC [mmol s^{-1} kg^{-1} (protein)] as indicated by the activity of the preparation after storage for 24 h. Means ± SE from five independent experiments.

Storage	Glycerol in the suspension medium	
	none	50 % (v/v)
Before storage	883 ± 13	900 ± 2
After storage for 24 h		
at 25-30 °C	22 ±17	250 ± 3
4 °C	138 ± 67	700 ± 13
-20 °C	267 ± 38	817 ± 22
in liquid nitrogen	450 ± 20	900 ± 28

Discussion

We report an efficient way to purify PEPC from light-adapted leaves of *A. hypochondriacus*. The high specific activity of 900 mmol s^{-1} kg^{-1}(protein) and yield of ~50 % reported here belong to the highest in literature (Toh *et al.* 1994, Chollet *et al.* 1996).

The activity of the enzyme varies depending on the type of extraction, pH, ionic strength, and salt used for elution. Phosphate was used as buffer for purification since it is a stabilizer/activator of purified PEPC from leaves of *A. viridis* (Podestá *et al.* 1990). Phosphate has also been used for elution of PEPC from soybean root nodules (Schuller and Werner 1993) and from leaves of maize (Hague and Sims 1980). PEPC from the leaves of *A. hypochondriacus* was eluted from HAP column, at P_i concentration of 60-80 mM. This is close to the range reported by Iglesias *et al.* (1986) for PEPC from *A. viridis* eluted also at 50-60 mM P_i. However, with sugarcane PEPC was eluted at 120 mM P_i (Iglesias and Andreo 1989). We obtained a high specific activity of the enzyme, the highest for any NAD-ME type C_4 plant. Specific activites of PEPC in the literature vary from low values of 67-170 mmol s^{-1} kg^{-1}(protein) to high values as 3000-3670 mmol s^{-1} kg^{-1}(protein) (O'Leary 1982). A reasonable range of average specific activity of purified PEPC (V_{max}) is about ~670 mmol s^{-1} kg^{-1}(protein) (Wang and Chollet 1993, Ogawa *et al.* 1997).

Table 4. Kinetic characteristics of purified PEPC after storage with 50 % (v/v) glycerol for 24 h at different temperatures. Means ± SE from five independent experiments.

Storage	V_{max} [mmol s^{-1} kg^{-1}(protein)]	K_m (PEP) [mM]	K_i (L-malate) [mM]	K_A (G-6-P) [mM]
Before storage	900 ± 3	0.44	0.5	0.3
After storage for 24 h at 4 °C	683 ± 33	0.27	7.0	2.3
-20 °C	750 ± 53	0.24	2.5	1.8
liquid nitrogen	900 ± 17	0.40	0.5	0.3

A single band of PEPC was observed when the SDS gel was loaded with either low (10 µg) or high (>30 µg) amounts of pure PEPC-protein (Fig. 2). On the other hand, two bands of PEPC appeared on the native gel electrophoresis and both stained for PEPC activity (Fig. 3). Therefore, the possibility of contamination with PPDK can be ruled out, as observed by Budde and Chollet (1986). We suggest that two bands of active PEPC in native-PAGE are presumably due to dissociation of tetramer to dimer at high pH in the gel. Their mobilities are too different from each other to be taken as isoforms. In fact, the activity stain is smeared between the two bands. Multiple forms of PEPC exist in leaf tissue of higher plants (Chen and Jones 1970, Mukerji 1977). However, there have been conflicting reports on the presence of isozymes. In maize leaves, a single band of 100 kDa protein was reported after SDS-tube gel electrophoresis (Uedan and Sugiyama 1976), whereas Hague and Sims (1980) observed two bands from maize on highly resolving SDS-PAGE slab gels.

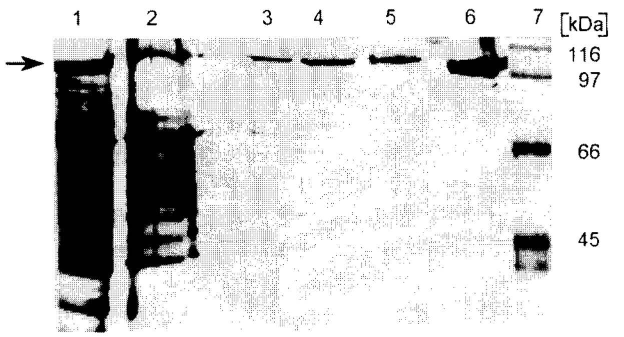

Fig. 2. Silver stained gels after SDS-PAGE of PEPC at different steps of purification from the leaves of *A. hypochondriacus*. Lane *1*: crude extract, lane *2*: 40-60 % saturated ammonium sulfate fraction, lane *3*: DEAE-*Sepharose* pool, lane *4*: hydroxylapatite pool, lane *5*: *Seralose 6-B* pool, lane *6*: purified PEPC after concentration with PEG-20 000 and storage in the presence of 50 % (v/v) glycerol, lane *7*: molecular mass markers (29-116 kDa). Lanes *1* and *2* each contained 10 µg protein, while lanes *3* to *6* each contained 4 µg protein. The *arrow* marks PEPC.

The kinetic characteristics of PEPC from leaves of C_3 and C_4 plants considerably vary (Andreo *et al.* 1987, Chollet *et al.* 1996). K_m for PEP and K_i for L-malate with the purified enzyme from *A. hypochondriacus* were around 0.4 and 0.5 mM, respectively (Table 2). This K_i value of the light-form of the purified enzyme is in agreement with the light-form of the enzyme reported in literature (Podestá *et al.* 1995). O'Leary *et al.* (1981) found a wide range of K_i values for L-malate for PEPC from various C_4 (0.04-10 mM) or CAM (0.006-6.200 mM) plants. Colombo *et al.* (1998) and Iglesias and Andreo (1989) reported a K_m (PEP) value of 0.29 mM in *A. viridis* and 0.25 mM for PEPC in sugarcane leaves.

Studies on the kinetics of PEPC before and after storage showed that its activity could be fully maintained only in the presence 50 % (v/v) glycerol at liquid

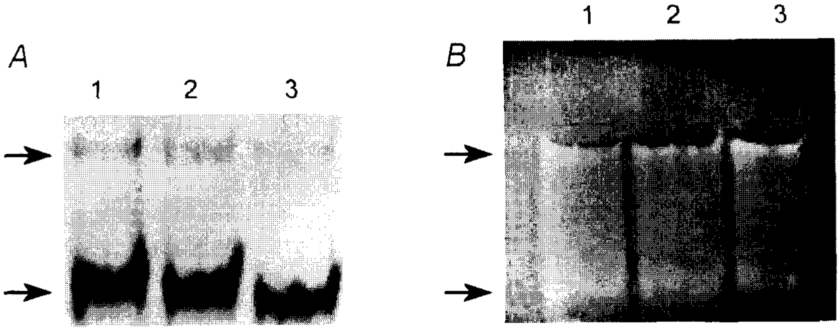

Fig. 3. Electrophoresis of purified PEPC on a nondenaturing 10 % polyacrylamide gel. (*A*) Gels stained with Coomassie brilliant blue R-250,. (*B*) gels stained for PEPC activity. 20 mg of purified PEPC was loaded in each lane.

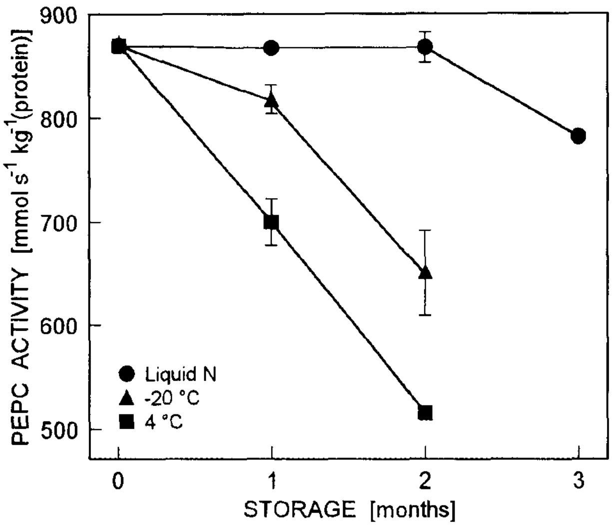

Fig. 4. The stability of PEPC purified from leaves of *A. hypochondriacus*. The enzyme was stored in either liquid nitrogen, a freezer (-20 °C), or a refrigerator (4 °C) in the presence of 50 % (v/v) glycerol. PEPC activity was assayed at pH 7.3 with 2.5 mM PEP. Values represent mean ± SE of the mean from five independent experiments.

nitrogen compared to 4 and -20 °C. Moreover, the enzyme could maintain its L-malate sensitivity as revealed by K_i value (Table 4) only in the presence 50 % (v/v) glycerol at liquid nitrogen temperature. The enzyme was stable for 2-3 months and this is of importance since it is thus easy to study the regulatory properties both *in vivo* and *in vitro* without losing its kinetic properties (Table 4). Several authors attempted PEPC storage in different ways: at 4 °C (Vidal *et al.* 1980, Jiao and Chollet 1988), -15 °C (Nimmo *et al.* 1986), -20 °C (Zhang *et al.* 1995), or in liquid nitrogen (Schuller and Werner 1993). Bakrim *et al.* (1992) showed that sorghum PEPC purified through an immunoadsorbent column lost its activity in the absence of glycerol when stored directly at 4 °C. PEPC from developing seeds of *Brassica campestris* was stored for one week at 4 °C (Mehta *et al.* 1995).

We found that addition of glycerol itself was enough to protect the enzyme during storage in a reasonably good way as indicated by low values of K_i (L-malate) and K_A (G-6-P) (Table 4). Glycerol acted as a cryoprotectant and addition of L-malate, PMSF, or G-6-P as suggested by other co-workers was not necessary. Addition of 5-10 mM L-malate or G-6-P has been recommended (Nimmo *et al.* 1986, Willeford *et al.* 1990, Zhang *et al.* 1995). Protease inhibitors such as PMSF or chymostatin have been included during storage by McNaughton *et al.* (1991) and Arrio-Dupont *et al.* (1992).

Our results indicate that simply the addition of glycerol was enough to maintain the activity of the enzyme at liquid nitrogen temperature. These observations confirm the good stability of PEPC in the presence of glycerol and inform that the high specific activity and yield can be obtained with PEPC of an NAD-ME type C_4-plant.

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