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Cloning, expression, purification and physical and kinetic characterization of the phosphoenolpyruvate carboxylase from orange (*Citrus sinensis* osbeck var. Valencia) fruit juice sacs

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ABSTRACT

Phosphoenolpyruvate (PEP) carboxylase (PEPCase) from orange fruit juice sacs has been cloned and heterogously expressed in high yield. The purified recombinant enzyme displays properties typical of plant PEPCase, including activation by sugar phosphates and inhibition by malate and citrate. Malate inhibition is weak in the physiological pH range, and the enzyme is also poorly affected by Glu and Asp, known inhibitors of C_3 plants PEPCases. However, it is strongly inhibited by citrate. Orange fruit PEPCase phosphorylation by mammalian protein kinase A decreased inhibition by malate. The enzyme presents an unusual high molecular mass in the absence of PEP, while in its presence it displays a more common tetrameric arrangement. The overall properties of the enzyme suggest that it is suited for organic acid synthesis and NADH reoxidation in the mature fruit. The present study provides the first analysis of a recombinant fruit PEPCase.

1. Introduction

PEPCase (EC 4.1.1.31) catalyzes the irreversible β-carboxylation of PEP according to the equation: PEP + HCO_3^- → oxaloacetate + Pi. The enzyme requires Mg^{2+} as an essential cofactor, being the only biotin-independent carboxylase employing HCO_3^-, rather than CO_2, as the substrate [1]. PEPCase is widely distributed among different organisms, including bacteria, protozoa, algae and plants, although it is absent in yeasts, fungi and animal tissues [1–3]. The enzyme plays a key role in photosynthetic cells of C_4 and CAM plants, where it participates in the initial fixation of atmospheric CO_2, catalyzing the first step of the so-called C_4 dicarboxylic acid pathway of photosynthesis [1,2]. In C_3 plants (and in general in heterotrophic plant tissues and organisms), the enzyme is situated at a major metabolic branchpoint [4]. Most relevant processes where PEPCase is involved include: (i) anaplerosis, by replenishing TCA cycle intermediaries, (ii) malate fermentation, (iii) nitrogen assimilation, (iv) maintenance of pH and electroneutrality, and (v) generation of NADPH [1–4].

PEPCases from photosynthetic tissues of C_4 plants and also from bacteria have been extensively characterized [1–3,5]. The 3D structure of the enzyme from *Escherichia coli* [3,6] and *Zea mays* [7] has been resolved by X-ray crystallography. Studies have also been performed to determine properties and metabolic roles played by the enzyme in green algae [8,9] and seeds [10–12]. Although many reports have pointed out the relevance of PEPCase in fruit tissue physiology, development and ripening [13–16], the enzyme from fruit has received scarce attention, as it has only been purified and characterized from avocado [17], banana [18], and cherimoya [19]. Given the high diversity in fruit physiology among species, there is a clear void of information on PEPCase biochemistry in these organs, despite the very important role that this enzyme has for carbon economy and organic acid production.

That PEPCase is important in citrus fruit physiology is evident in a recent work carried out in our laboratory [14], in which it was demonstrated that PEPCase activity is significantly increased in orange fruits exposed to freezing injury. This result prompted us to obtain molecular tools to better identify the molecular scenario operating in the fruit tissue under physiological and stress conditions and in particular to examine the properties of orange fruit

Abbreviations: CAM, Crassulacean Acid Metabolism; *Csi*PEPCase, *Citrus sinensis* phosphoenolpyruvate carboxylase; Fru-6-P, fructose-6-phosphate; Glc-6-P, glucose-6-phosphate; PEP, phosphoenolpyruvate; PEPCase, phosphoenolpyruvate carboxylase; PKA, protein kinase A; TCA, tricarboxylic acid cycle.

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Table 1
Oligonucleotides utilized for the cloning of the gene coding for PEPCase from orange fruit. Restriction sites are underlined.

Name	Sequence	Restriction site
*Csi*PEPC-Fo	CATATGGCGGCGAATAGGAATTTAGAG	NdeI
*Csi*PEPC-Re	GTCGACTTAACCAGTGTTTTGC	SalI
*Csi*PEPC-int1	CTGGTCTTGACTGCTCATCC	–
*Csi*PEPC-int2	GGATGAGCAGTCAAGACCAG	–
*Csi*PEPC-int3	CTGAACTTAGAGGCAAACGTCC	–
*Csi*PEPC-int4	GGACGTTTGCCTCTAAGTTCAG	–
*Csi*PEPC-int5	CACCAAAACCAGAATGGCGTG	–
*Csi*PEPC-int6	CACGCCATTCTGGTTTTGGTG	–

PEPCase (*Csi*PEPCase) and how these are related to the general carbon metabolism in orange fruit. Given the difficulties in isolating the enzyme from juice sacs, the cloning and expression was chosen as the source of active enzyme. In this work we report on the development of a strategy to accomplish the molecular cloning of the gene coding for PEPCase in fruits of *Citrus sinensis* var. Valencia late. We constructed an expression vector to produce the enzyme as a recombinant fusion protein. Results show the convenience of the system to obtain the *Csi*PEPCase in an active form, as well as in preparative amounts and as a highly purified protein to perform kinetic and structural studies. To the best of our knowledge, this is the first time that a PEPCase from fruit is obtained recombinantly.

2. Materials and methods

2.1. Plant material, bacterial strains and chemicals

"Valencia" orange fruit (*C. sinensis* L. Osbeck) were harvested from trees of selected lots of the INTA's Estación Experimental Agrícola Concordia (Entre Ríos, Argentina). Freshly collected fruits were frozen in liquid nitrogen and stored at −80 °C until use. *Escherichia coli* TOP10 (Invitrogen) cells were used for cloning procedures and plasmid maintenance. Protein expression was carried out with *E. coli* BL21 Star (DE3) (Invitrogen). Cells were grown in Luria Bertani medium supplemented with ampicillin (100 μg/ml) when necessary. PEP, L-malate, Glc-6-P, NADH and pig heart malate dehydrogenase were from Sigma–Aldrich. All other chemicals were of the highest purity available.

2.2. Cloning of CsiPEPCase

Total RNA from orange fruit pulp (10 months after pollination) was isolated as described by Hall et al. [20] and mRNA was purified with the PolyATtract mRNA Isolation System IV (Promega). cDNA was synthesized at 42 °C for 1 h in a 25 μl reaction mixture containing 0.25 μg of mRNA, 200 pmol of oligo(dT) primer and 200 U of M-MLV reverse transcriptase (USB). To amplify the gene encoding for PEPCase from orange pulp, we used specific primers (see Table 1) based in the information available in the HarvEST:Citrus 1.20 program (http://harvest.ucr.edu/). PCR reaction was performed in a 50 μl reaction mixture containing 2 μl of cDNA solution, 100 pmol of each primer and 2.5 U of *Taq* DNA polymerase (Fermentas). PCR conditions were one cycle of 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 30 s at 50 °C and 1 min at 72 °C, followed by a final cycle of 10 min at 72 °C. Four DNA fragments were amplified and used to obtain the full-length coding sequence of *Csi*PEPCase combining PCR and digestion with restriction enzymes and ligation with T4 DNA ligase (Promega). The amplified gene was cloned into the pGEM-T Easy vector (Promega) and its identity was confirmed by automated sequencing (Macrogen). These DNA fragment was subcloned into the pET32a vector (Novagen) to obtain the recombinant protein fusioned to *E. coli* Trx and a His-tag in the N-terminus as previously described [21]. The construction [pET32a/*Csi*PEPCase] was used to transform *E. coli* BL21 Star (DE3) cells (Invitrogen) in order to express the recombinant enzyme.

2.3. Expression and purification of recombinant PEPCase

Transformed colonies bearing the [pET32a/*Csi*PEPCase] plasmid were grown at 37 °C in 3 ml Luria Bertani medium plus 100 μg/ml ampicillin with constant shaking for 16 h. From the latter suspension, a volume enough to achieve an absorbance of 0.05 was inoculated to 50 ml of LB medium containing the specified antibiotic. The culture was incubated with constant shaking at 30 °C, and growing was followed by measuring absorbance at 600 nm in a UNICAM, HEλI0S spectrophotometer. When the optical density reached a value of ~0.6, expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (to a concentration of 200 μM) followed by overnight incubation at 16 °C. Final optical density was measured and aliquots were taken before centrifuging at 4000 × *g* for 5 min at 4 °C. The cell paste was resuspended in lysis buffer (20 mM Tris–HCl, 0.5 M NaCl, 20 mM imidazole, 5% (v/v) glycerol, 0.1% (v/v) Triton X-100, 20 mM KCl, 10 mM $MgCl_2$, pH 7.5) plus 1 mM PMSF and 0.1 mM E-64. The volume of lysis buffer utilized was calculated considering: lysis buffer added (μl) = final optical density × 100 × total cell culture volume (ml) before centrifugation.

Cells were disrupted in an MSE sonicator by applying 8 × 15 s pulses at maximum power, with 1 min intervals, maintaining the sample on a water-ice bath. Cell extracts were centrifuged for 20 min at 4500 × *g*, at 4 °C. Both fractions, the supernatant (soluble fraction) and the pellet (insoluble fraction) were kept at −20 °C until use. The supernatant was further processed for protein purification using a 1 ml His-Trap column (GE Healthcare). The lysate supernatant and the buffers used were filtered through a 0.2 μm Minisart (Sartorius) filter. The column was previously treated by washing with 10 column volumes distilled water and equilibrated with identical volume *buffer A* (20 mM Tris–HCl, 0.5 M NaCl, 20 mM imidazole, 5% (v/v) glycerol; 20 mM KCl, 10 mM $MgCl_2$, pH 7.5). The supernatant was loaded and the column, washed with 5 bed volumes of *buffer A* and then with 5 volumes of the same buffer but containing 50 mM imidazole. Bound protein was eluted in a single step with 5 volumes of *buffer A* where concentration of imidazole was increased to 300 mM. Alternatively, gradient elution from 50 to 500 mM imidazole was performed using an ÄKTA™ purifier (GE Healthcare), looking to improve purification performance in this initial step. Aliquots were of 1 ml during loading and washing and 0.5 ml during elution. Fractions were analyzed by SDS-PAGE, and measurements of total protein and PEPCase activity.

The immunological identity of the recombinant protein was confirmed by western blotting using antiserum raised against the *Amaranthus viridis* PEPCase [22]. Purified active fractions were pooled and concentrated in Amicon Ultra-15 (Millipore) tubes with an exclusion limit of 30 kDa by spinning at 2000 × *g* and 4 °C. Two buffer changes were made during sample concentration, using 5 volumes each of proteolysis buffer (20 mM Tris–HCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 8.0). Finally, glycerol was brought to 20% (v/v) and the samples were stored at −20 °C. Under these conditions the recombinant enzyme remained fully active for at least 3 months.

2.4. N-terminal digestion of the fusion protein

Removal of the His-tag in the recombinant protein was achieved by treatment with enterokinase (New England BioLabs) in proteolysis buffer (see above). Optimal conditions were determined by assaying different temperatures, digestion times and protease:fusion protein ratios. The proteolytic reaction was stopped with 5 mM p-amino benzoic acid.

2.5. Protein determination

Protein concentration was measured using the BCA™ Protein Assay Kit (Pierce) [23]. BSA was used as a standard.

2.6. Enzyme activity

*Csi*PEPCase activity was followed by measuring the decrease in absorbance at 340 nm, at 30 °C, by coupling PEPCase reaction to malate dehydrogenase, in a medium containing 100 mM Tris–HCl, 10% (v/v) glycerol; 10 mM $MgCl_2$; 0.15 mM NADH; 3 U pig heart malate dehydrogenase; 10 mM $NaHCO_3$ and 4 mM PEP (monocyclohexyl-ammonium salt) at pH 7.0 or 8.0 and an adequate amount of enzyme. One unit (U) is defined as the amount of enzyme catalyzing the consumption of 1 μmol/min of substrate under the specified conditions.

Estimation of the kinetic parameters was performed by varying PEP concentration at fixed levels of Mg^{2+} and $NaHCO_3$. Data were fitted to the Michaelis–Menten equation by non-linear regression using the Kinetics computer program [24]. It was checked that activity was proportional to the amount of enzyme utilized and that it followed Michaelian kinetics with the same program by analyzing fitting accuracy to the Hill equation. The enzyme preparations gradually lost activity and were less stable in the reaction medium unless they were preincubated with 1 mM PEP. Kinetic constants are the mean of at least three independent data sets and they were reproducible within ±10%.

To analyze the effect of different metabolites on *Csi*PEPCase activity, fixed amounts of Glc-6-P, malate and the other compounds were added to a cuvette in which the reaction had been started. The I_{50} value (amount of effector giving 50% inhibition) for malate was determined by adding varying amounts of the metabolite to a cuvette under subsaturating PEP concentrations. The I_{50} was calculated using the specified kinetics program [24]. To test the effect of Glc-6-P, the concentration of this activator was varied at constant concentration of substrates and the K_a was calculated. This value represents the concentration of activator that gives half-maximum activation at a fixed concentration of substrate.

2.7. Electrophoretic methods

PAGE under native or denatured (SDS-PAGE) conditions were performed essentially as described by Bollag and Edelstein [25] using a Mini-Protean III gel electrophoresis apparatus (Bio-Rad). Non-denaturing separating gels were at a pH of 8.8. To detect PEPCase activity after native PAGE gels were equilibrated in 100 mM Tris–HCl, 20% (v/v) glycerol and 20 mM $MgCl_2$ at pH 8.0 for 10 min at room temperature. Gels were then transferred to a medium containing 100 mM Tris–HCl, 1 mM EDTA; 10% (v/v) glycerol, 20 mM $MgCl_2$, 50 mM $NaHCO_3$ and 5 mM PEP at pH 8.0 during 45 min at 30 °C. Next, gels were transferred to a 3 mg/ml Fast Violet B salt solution and stirred in the dark until red bands were visible; upon which fixation was accomplished with 5% (v/v) acetic acid, previous washing with distilled water.

2.8. Size exclusion chromatography

Analytical size exclusion chromatography was performed using an ÄKTA purifier (GE Healthcare) to which a Sephacryl S-300 (22 cm × 16 mm) or a Phenomenex BIOSEP SEC S3000 (300 mm × 7.8 mm) were attached. Columns were equilibrated with 20 mM Tris–HCl, 10% (v/v) glycerol, pH 7.5 and, where indicated, with the addition of 0.5 mM PEP or 5 mM malate. Samples or mass standards (thyroglobulin (669 kDa); apoferritin (440 kDa); amylase (232 kDa); alcohol dehydrogenase (140 kDa) and BSA (66 kDa), obtained from Sigma-Aldrich, were applied with a volume of 100 μl and run at a constant flux of 0.7 ml/min.

2.9. Preparation of antibodies against CsiPEPC

Polyclonal antibodies against the recombinant *Csi*PEPCase were obtained by immunization of rabbits with 400 μg of the purified protein in two subcutaneous injections of 200 μg each performed at different intervals from the extraction of the preimmune serum. The antibodies against the recombinant *Csi*PEPC were further purified from the crude antiserum [26].

2.10. In vitro phosphorylation of CsiPEPC

The phosphorylation of purified recombinant *Csi*PEPC by PKA was performed in a reaction mixture (15 μl in total volume) consisting of 20 mM Tris–HCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM (185 kBq) [γ-^{32}P]-ATP (GE Healthcare Life Sciences), 5 μg purified PEPC and 20 units of PKA (catalytic subunit, Sigma) at pH 7.5, incubated at 30 °C for 40 min. The reaction was terminated by addition of SDS sample loading buffer and then subjected to SDS-PAGE, dye-staining, and autoradiography (Phosphorimager Storm 860; Molecular Dynamics).

3. Results

3.1. Cloning, expression and purification of CsiPEPCase

As no information is available about the sequence of the gene coding for *Csi*PEPCase, a search for ESTs was conducted in the HarvEST:Citrus 1.20 program. The ESTs were aligned with known PEPCase sequences from other plant species (data not shown) and those highly conserved regions were used to design specific primers (see Table 1). Using cDNA from orange pulp and the specific primers we amplified four DNA fragments (1, 2, 3, and 4; see Fig. 1) in the first PCR round. Each fragment exhibited a unique sequence, with exception of fragment 2, which presented two different sequences of the same length. Of these, only one produced an ORF homologous to PEPCs when it was assembled with the other three fragments. As their ends were complementary, the fragments were joined in a second PCR to obtain three larger DNA fragments (called I, II, and III; see Fig. 1). Finally, each of these sequences was digested with specific restriction enzymes (BglII for I and II, and HindIII for II and III; these sites were present in the sequence of the respective fragment, each one as a single site per fragment; see details in Fig. 1). The complementation was utilized to join the fragments with T4 DNA ligase to obtain a full-length coding sequence of 2904 bp. This novel complete sequence has been submitted to the NCBI (GenBank accession no. EF058158.2). The cloned gene encodes for a putative protein of 967 amino acids of calculated molecular mass 110,754 Da and *p*I 6.21. An alignment of the deduced amino acid sequence of *Csi*PEPCase with PEPCases from different sources depicted in Supplementary Fig. 1 indicates that the orange fruit enzyme is structurally more related to the PEPCase from plants (identity 79% or higher) than to the enzyme from bacteria (identity ~37%). Furthermore, *Csi*PEPCase exhibits structure domains typical of plant-type PEPCases: (i) the invariant QNTG C-terminal sequence [27], and (ii) the conserved N-terminal seryl-phosphorylation site (EKMAS̲IDAQLR) [12]. Also from Supplementary Fig. 1, it can be inferred that *Csi*PEPCase is similar to the C_3-type PEPCase respect to the presence of Ala at position 776, which in the C_4-type enzyme is replaced by Ser [2,28,29]. Furthermore, the phylogenetic tree depicted in Supplementary Fig. 2, where the *Csi*PEPCase was compared with 18 full-length PEPCase sequences obtained from GenBank, reveals that the *Csi*PEPC gene shares a common ancestor with the homotetrameric PEPC1

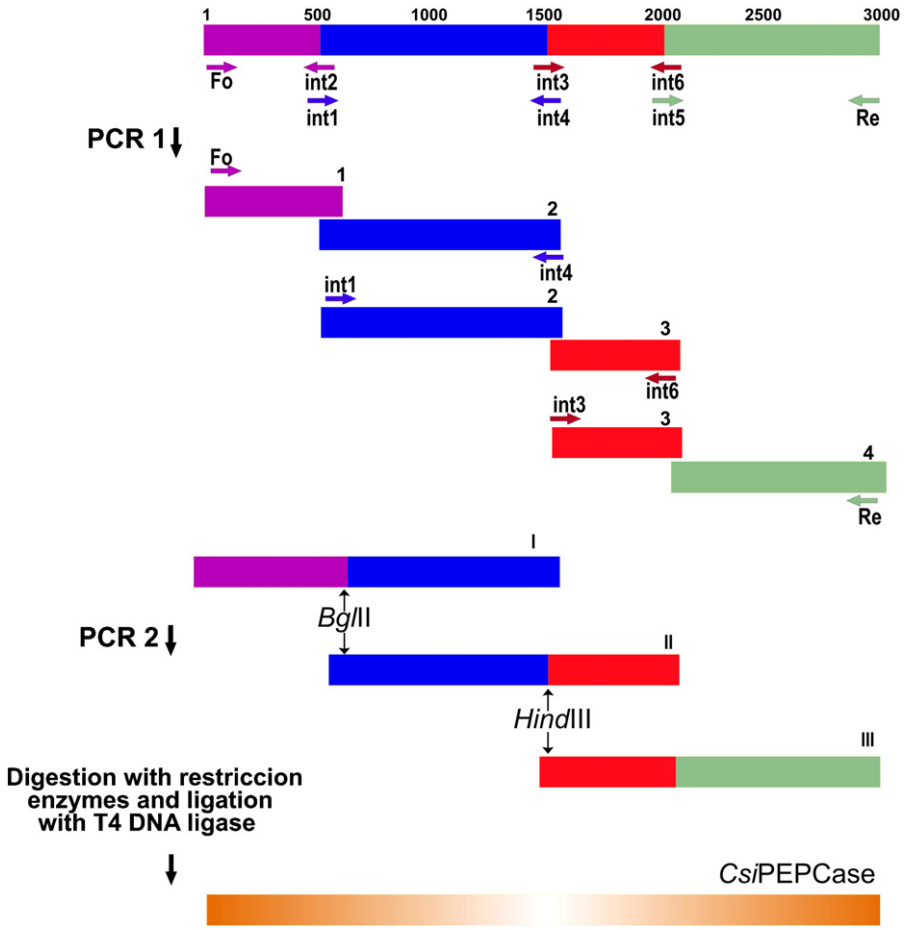

Fig. 1. Schematic representation of the strategy developed to clone the gene coding for PEPCase from orange fruit.

from *Ricinus communis* [30] and with the most abundant isoform in soybean root nodules [31].

After induction, transformed *E. coli* cells showed high levels of a 127 kDa polypeptide which was recognized by anti-*A. viridis*-PEPCase IgG (Fig. 2A and B). Following purification of recombinant *Csi*PEPCase by Ni^{2+} chelation chromatography, the enzyme was subjected to controlled proteolysis with enterokinase, yielding a 110 kDa polypeptide (Fig. 2B). The recombinant *Csi*PEPCase showed a high activity, displaying a specific activity of 18 U/mg protein at pH 8.0. Immunopurified antibodies raised against the recombinant enzyme recognized the *Csi*PEPCase protein as a unique band in western blots of juice sacs crude extracts (not shown).

3.2. Molecular properties of recombinant CsiPEPCase

Fig. 3 shows a non-denaturing PAGE of purified recombinant *Csi*PEPCase before and after digestion with enterokinase, in addition to PEPCase from different plant extracts. In all cases, *Csi*PEPCase migrated with an apparent mass higher than the heaviest (669 kDa) marker, while the maize enzyme migrated as a band slightly below 232 kDa. Interestingly, the higher mass isoform of *R. communis* germinating endosperm PEPCase, of molecular mass around 680 kDa [10], migrated in a similar way than that of the orange fruit enzyme. Similar results were obtained when the enzyme was immunodetected after non-denaturing PAGE of crude extracts (Fig. 3B). These results were confirmed by size exclusion chromatography, where *Csi*PEPCase eluted very close to the void volume and before the highest mass standard, thus ruling out an abnormal mobility of *Csi*PEPCase during gel electrophoresis (Fig. 4). Nevertheless, a

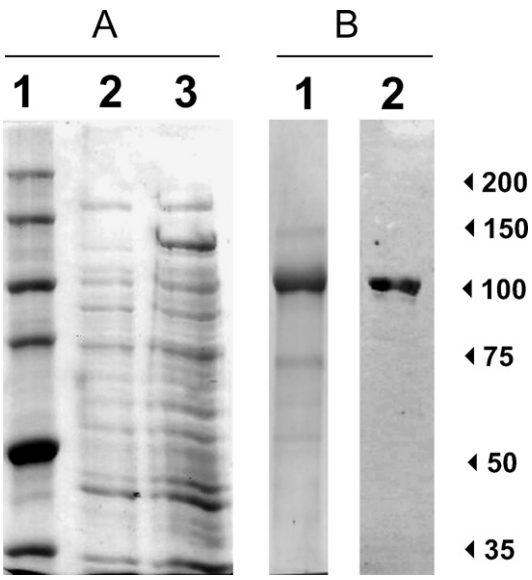

Fig. 2. Expression and purification of *Csi*PEPCase. (A) SDS-PAGE. Lane 1: molecular mass markers; lane 2: extract from transformed *E. coli* BL21 Star (DE3) cells before induction; lane 3: extract from transformed *E. coli* BL21 Star (DE3) cells after induction. (B) Lane 1: SDS-PAGE of recombinant purified, His tail excised, *Csi*PEPCase; lane 2: Western blot of the same sample.

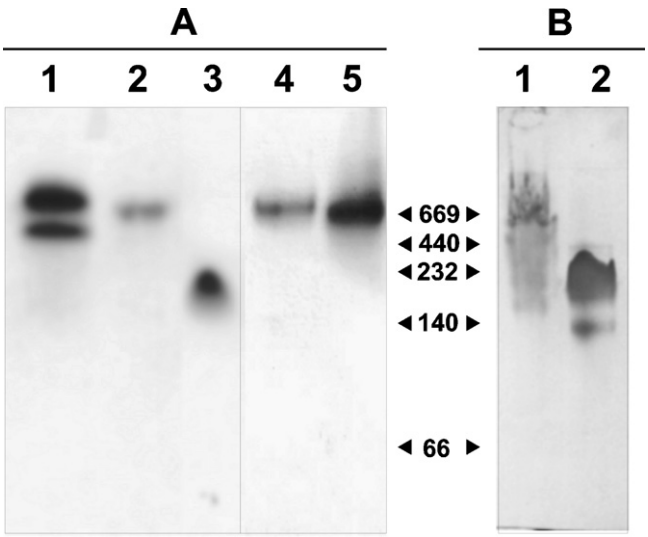

Fig. 3. Molecular structure characterization of PEPCase. (A) Activity determination in non-denaturing gels. Lane 1: endosperm of developing (stage VI) *Ricinus communis* seeds extract; lane 2: orange fruit endocarp clarified extract; lane 3: maize leaf extract; lane 4 and 5: purified recombinant PEPCase before and after digestion with enterokinase, respectively. Lane 3 was run in the same gel as lanes 1 and 2, cut and moved for clarity of the figure. Lanes 4 and 5 pertain to a different gel run under the same conditions. (B) Western blot of a PAGE under native conditions. Lane 1: orange fruit endocarp extract, lane 2: maize leaf extract. Anti-*A. viridis*-PEPCase antibodies were used.

shoulder at a region where the tetramer would presumably elute is present in the trace. Interestingly, when the enzyme was chromatographed in a buffer containing 0.5 mM PEP, a displacement was observed in the peaks in a way that a small shoulder appeared close to the void volume, whereas a major peak appeared at around 440 kDa. Malate did not substantially change the elution profile of *Csi*PEPCase. From these experiments it can be concluded that both the recombinant and native *Csi*PEPCase exist as a high molecular mass aggregate (probably in a hexameric or octameric arrangement of subunits) in the absence of PEP. In the presence of the substrate, the structure is displaced to a more common tetrameric arrangement. Contrarily, malate showed no effect on the enzyme's

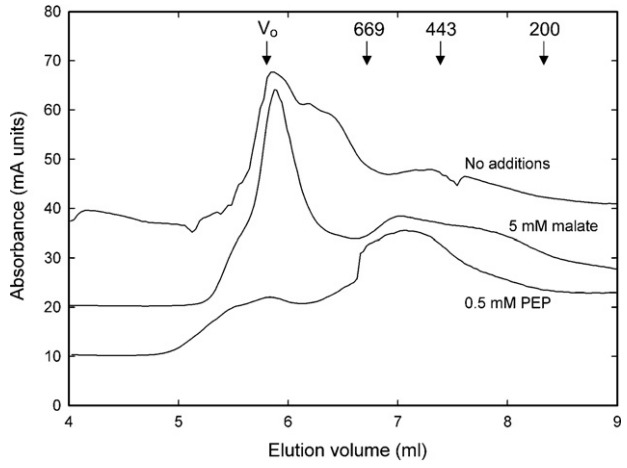

Fig. 4. Elution profile of *Csi*PEPCase after size exclusion chromatography column. The enzyme was applied to the column in a buffer that contained no additions (upper trace), 5 mM malate (middle trace) or 0.5 mM PEP (lower trace), as described in Section 2.8. Five micrograms of *Csi*PEPCase (specific activity 18 U/mg) were loaded for each run. The arrows indicate the void volume (V_o) and the elution volume of the standards. Numbers above the arrows represent the respective mass (in kDa) of each marker.

Table 2
Kinetic parameters of purified recombinant *Csi*PEPC. Each value represents the average of three separate measurements.

Parameter	pH 7.0	pH 8.0
K_m (PEP) (μM)	79 ± 14	133 ± 14
V_{max} (unit mg^{-1})	16.7 ± 0.6	17.8 ± 0.4

quaternary structure. It is interesting to note that PEPCase activity showed instability (gradual loss of activity) unless previously incubated with PEP before assay. These two findings underscore the role of PEP in the maintenance of *Csi*PEPCase in an active conformation.

3.3. Kinetic properties of CsiPEPCase

The purified recombinant *Csi*PEPCase activity was pH-dependent in the usual manner for all plant PEPCases, exhibiting a bell-shaped curve centered at pH 8.0, declining rapidly below pH 7.5. Activity at pH 7.0 was 75% of that observed at pH 8.0, in good agreement with the values reported for the activity in crude extracts from orange fruit [14]. *Csi*PEPCase K_m for PEP was in the low micromolar range, showing the enzyme more affinity at pH 7.0 than pH 8.0 (Table 2). Saturation curves for PEP were hyperbolic in both cases (Fig. 5).

A range of metabolites were tested as possible effectors of the enzyme, at saturating and subsaturating levels of PEP. In common with most PEPCases, Glc-6-P activated the enzyme at low but not high PEP levels, and only at pH 7.0, nearly doubling activity. The K_a, obtained at a PEP concentration close to its K_m, was 0.14 ± 0.03 mM and hyperbolic kinetics were observed for Glc-6-P binding. The same effect was caused by Fru-6-P and to a lesser extent by glycerol-3-P (Table 3). Succinate, citrate, ATP and the amino acids Asp and Glu inhibited *Csi*PEPCase, in all cases more pronouncedly at pH 7.0. Malate, a known effector of most PEPCases, acted in a competitive fashion with respect to PEP, with a K_i of 2.7 mM at pH 7.0 (Fig. 6), as judged from double reciprocal plots obtained at different malate concentrations (not shown) and confirmed from tertiary plots (inset in Fig. 6). The enzyme was slightly more inhibited at pH 8.0 than at pH 7.0, with an I_{50} of 3.6 and 5.0 at each pH value, respectively (Table 3).

In the presence of malate the enzyme was activated by Gc-6-P to a much lesser extent than in its absence: 128% versus 198%, respectively. On the other hand, when malate was added at an I_{50} concentration in the presence of Glc-6-P, the residual activity was

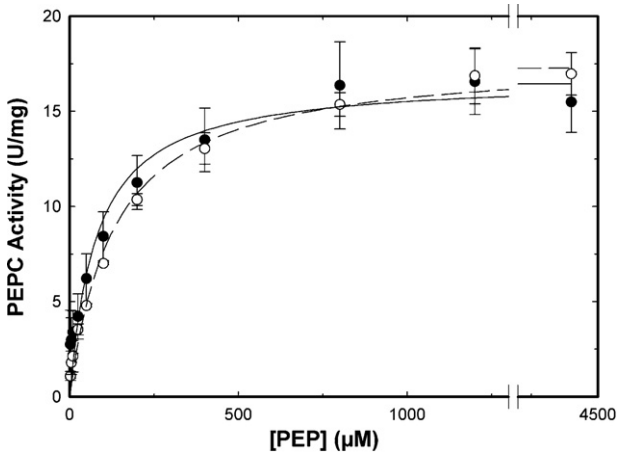

Fig. 5. Kinetics of saturation by PEP of recombinant CsiPEPCase. Activity was determined at pH 7.0 (closed circles) or 8.0 (open circles).

Table 3
Effect of different metabolites on *Csi*PEPCase. PEPC activity was determined at pH 7.0 or 8.0 with subsaturating PEP (80 and 130 μM, respectively) and with saturating PEP (4 mM) in the presence and absence of each effector at 2 mM. Activities are expressed relative to the respective control determined in the absence of any additions and set at 100%. Shown in parentheses are the K_a (Glc-6-P) or I_{50} (malate) values. All values represent the mean of three separate determinations and are reproducible within ± 10% (S.E.) of the mean value.

Effector	Relative activity (pH 7.0)		Relative activity (pH 8.0)	
	Subsaturating PEP	Saturating PEP	Subsaturating PEP	Saturating PEP
Malate	70.5 (I_{50} = 5.0 mM)	98.6	62.6 (I_{50} = 3.6 mM)	100.1
Glc-6-P	189.7 (K_a = 0.14 mM)	100.9	106.7	105.9
Fru-6-P	183	99	109	102
Glycerol-3-P	166	97	107	99
Succinate	67	87	69	91
Citrate	34	87	74	105
Asp	76	92	93	94
Glu	72	85	93	98
Ala	92	102	94	103
ATP	51	85	84	104

28.7%. These data indicate that the action of malate is to render an enzyme form less susceptible to Glc-6-P but also that it acts as a better inhibitor in the presence of Glc-6-P, suggesting that the change elicited by malate is such that it cannot be completely overcame by Glc-6-P. Ala did not have a noticeable effect on *Csi*PEPCase activity.

3.4. Phosphorylation of recombinant PEPC with PKA

As mentioned in Section 3.1, the deduced amino acid sequence of the orange PEPC indicated that there is a plant PEPC-invariant phosphorylation domain at its N-terminus. Ser-7, located in the (E/D)(K/R)xxSIDAQ(L/M)R motif, is presumably the target residue for regulatory phosphorylation. On this basis, we conducted an *in vitro* phosphorylation experiment with recombinant PEPC, using the mammalian PKA, whose target sequence is contained in the motif described. Fig. 7 illustrates that, effectively, the orange PEPC could be phosphorylated by PKA. The phosphorylated enzyme assayed at pH 8.0 showed a slightly lower sensitivity towards malate, as denoted by an I_{50} of 4.1 ± 0.6 mM versus 3.6 ± 0.3 mM for the control.

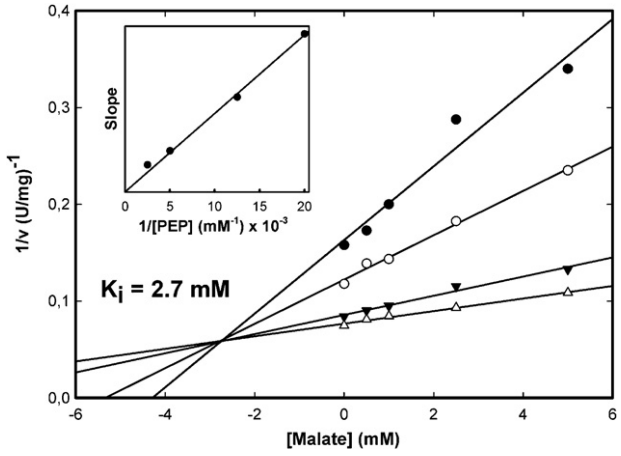

Fig. 6. Dixon-plot determination of K_i (malate) for the recombinant *Csi*PEPC at pH 7.0. Activity was measured in the presence of 100 mM Tris–HCl, 10% (v/v) glycerol; 10 mM $MgCl_2$ and 10 mM $NaHCO_3$. Inset: slope of the secondary graph versus the reciprocal of the substrate concentration. *Csi*PEPCase activity was determined with varying malate levels, as shown in the plot, at different PEP concentrations, as follows: 50 μM (closed circles), 80 μM (open circles), 200 μM (closed triangles) and 400 μM (open triangles).

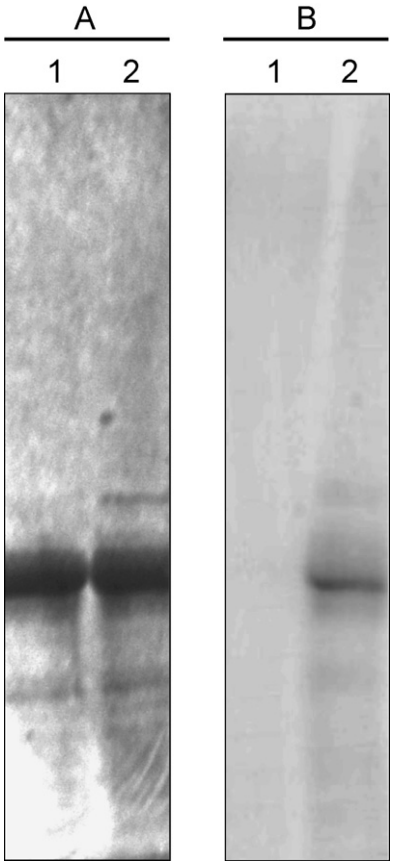

Fig. 7. *In vitro* phosphorylation of recombinant PEPC by mammalian PKA. (A) Coomassie blue-stained gel; (B) corresponding autoradiogram. Lane 1: purified recombinant PEPC (5 μg); Lane 2: purified recombinant PEPC (5 μg) after phosphorylation experiment.

4. Discussion

The citrus hesperidium is a fleshy fruit characterized by high sugar and organic acids content, whose levels and ratio are developmentally controlled. Edibility and palatability are dependent on a delicate balance between acidity and sugar content and constitute therefore a very important commercial issue. Malic and citric acids, mostly restricted to the vacuole, account for the majority of the organic acids content in citrus fruit [32]. Their synthesis during development is performed by glycolytic degradation of sucrose, in a process where PEPCase plays a key role [32,33]. Among the many

faceted functions of PEPCase in heterotrophic tissues, it has been linked to organic acid production in many plant tissues, from roots to fruits, due to its capacity to provide intermediaries to the citric acid cycle [4]. The extensive literature on PEPCase is remarkably deficient in the study of its function in fruit; although it has been partially characterized in tomato, avocado, grape and cherimoya [17,19,34,35], only in banana has a thorough study, including its phosphorylation, been completed [18,36]. In this paper we report on the cloning, expression and biochemical characterization of orange fruit PEPCase and discuss its probable implication in carbon economy.

Previous results show that the activity of PEPC increased in frost-exposed orange fruit with respect to a non-exposed control, while that of pyruvate kinase, which catalyzes a reaction at the same level, was not affected [14]. This fact, in addition to the lowered sensitivity of PEPC to inhibition by malate in exposed fruit, was explained in the context of an elevated fermentative metabolism because, in combination with cytosolic NAD-malate dehydrogenase, *Csi*PEPCase can be used to regenerate NAD^+, thus acting as an ancillary fermentation enzyme [14]. While it became evident that the elucidation of the role of *Csi*PEPCase in citrus fruit required an in-depth study of the isolated enzyme, it was also clear that the characteristics of the citrus fruit would hinder any attempt of purification of the enzyme directly from the fruit, mainly due to the highly aqueous and very acidic nature of the tissue and the high pectin content. As a result, the work was aimed at cloning and expressing *Csi*PEPCase in a heterologous system.

In the work described here, a full-length cDNA coding for *Csi*PEPCase was isolated from orange pulp using an effective strategy based in the highly conserved regions of known PEPCase sequences from different plant species. The obtained sequence showed high similarity with different PEPCases and the recombinant protein presented a high specific activity. Amino acid sequence analysis showed that the domains involved in catalysis and regulation are well conserved in *Csi*PEPCase. In addition, the sequence of the orange fruit enzyme is significantly more related to plant PEPCases. It also has specific domains typical of the enzyme from plants, as the N-terminal seryl-phosphorylation region and a characteristic C-terminus. Although the high sequence identity makes the establishing of a distinction between PEPCases from a C_3- or a C_4-plant difficult [2,28,29], *Csi*PEPCase has an Ala residue at position 776, which is highly conserved in the enzyme from C_3-plants but replaced by Ser in the C_4-PEPCases. Furthermore, a phylogenetic analysis (Supplementary Fig. 2), shows that *Csi*PEPCase shares a common ancestor with the homotetrameric PEPC1 from *Ricinus communis* [30], a typical housekeeping PEPCase.

The examination of the kinetic and regulatory properties of recombinant *Csi*PEPCase threw results which are consistently similar to those observed for other plant PEPCases. An optimum pH of 8.0 was found in the pH range 5.5–9.0, at saturating substrate concentrations. This optimum pH value was similar to those reported for PEPC in avocado [17], banana [18], cherimoya [19] and in orange extracts [14]. In common with most plant PEPCases, the recombinant enzyme displayed hyperbolic PEP saturation kinetics at pH 7.0 and 8.0, with K_m values of the same order that in crude extracts from citrus fruit [14] and comparable to those described in cherimoya fruit and other fruits like avocado or banana, but lower than in maize leaf (a C_4-species) [17–19,37].

*Csi*PEPCase was inhibited by malate and citrate and activated by Glc-6-P. Malate inhibition is relatively weak compared with its photosynthetic counterparts from C_4 plants or other housekeeping C_3-PEPCases. The I_{50} is higher at pH 7.0 than 8.0 (5.0 mM versus 3.6 mM, respectively). These values in the high millimolar range are uncommon for a plant PEPCase. It is also notable that *Csi*PEPCase activity is lightly affected by Glu and Asp, two known allosteric inhibitors of C_3-PEPCases [10,38,39]. The lack of regulation by Glu and Asp suggests that there is little, if any, crosstalk of carbon and N metabolism in orange at this level. The low malate sensitivity of PEPCase in orange fruit suggests that it would be more suited for the synthesis of malate. *Csi*PEPCase was phosphorylated by mammalian PKA and this modification altered, albeit slightly, malate sensitivity. Blonde and Plaxton [10] have argued that the presence of two PEPCase isoforms in *R. communis* with different malate, Asp and Glu sensitivities reveals the use of each isozyme for either organic acid or amino acid synthesis.

So far, there is no evidence about the existence of another PEPCase isoform being expressed in citrus fruit, because both PAGE and IEF under native conditions performed with fruit extracts of different developmental stages showed a unique band in all cases (Supplementary Figs. 3 and 4). Furthermore, an examination of all the PEPCase peptide sequences reported for the mature citrus fruit proteome [40] reveal that they correspond to a single sequence that matches the *Csi*PEPCase sequence described here.

Mature citrus fruit show a decreased aerobic respiratory metabolism and in fact ethanol is accumulated in the last stages or postharvest, an indication of a switch towards fermentative metabolism [32,41]. Similarly, frost-exposed orange fruit shows a dramatic decrease in respiration, accumulation of fermentation products and induction of fermentative enzymes [14]. It has been proposed that the increase in PEPCase activity and changes in its properties (i.e. less malate sensitivity) upon frost exposure are the result of the *Csi*PEPCase's action as an ancillary fermentative enzyme that oxidizes NADH back to NAD^+ to allow the continuation of glycolysis and maintenance of ATP levels [14]. The *Csi*PEPCase subject of this study portrays an inhibition profile by metabolites which suggest that it is suited for two main roles through the fruit development and ripening: citric acid synthesis in the early stages and reoxidation of NADH during the last stages or postharvest.

The *Csi*PEPCase-dependent acid accumulation/synthesis is under regulation since malate and citrate do inhibit the enzyme. Even if the extent of inhibition is not too high (70% and 34% residual activity at 2 mM of malate and citrate, respectively), the concentration of the two acids can be very high in the cytosol, as confinement to the vacuole is only 70% for malate and 89% for citrate [32]. Therefore, cytosolic concentrations of both compounds can reach levels that are high enough to limit activity. In mature fruit, citrate is moved from the juice sacs to the peel where it is metabolized [32]. Any imbalance in this transport would curtail *Csi*PEPCase's activity. Also, temperature fluctuations affecting the tonoplast permeability can affect PEPCase activity by altering the distribution of malate and citrate between the vacuole and the cytosol. In contrast to other PEPCases [39], inhibition by malate was not relieved by the addition of Glc-6-P. Moreover, the presence of the inhibitor almost completely negated PEPC activation by Glc-6-P. Neither malate nor Glc-6-P modified the hyperbolic kinetics. Whatever the mechanism of interaction between both effectors, it is clear from our results that Glc-6-P by itself is a very inefficient activator of (or it does not bind at all to) *Csi*PEPCase if malate is present. A similar behavior was observed in kinetic studies performed on the enzyme from maize [42].

Regarding the structural properties of *Csi*PEPCase, it presented an unusual (for homomeric PEPCases) high apparent mass observed by both non-denaturing PAGE and size exclusion chromatography. These results are not the outcome of an artifact provoked by high protein concentration or improper folding of the recombinant enzyme, as the same pattern is observed in crude extracts or partially purified *Csi*PEPCase. More interesting still was the fact that the presence of PEP in the chromatography buffer caused the appearance of a major peak at around 440 kDa, indicating the existence of the more common tetrameric arrangement of PEPCase subunits. Previous studies have shown that C_4-PEPC from maize exists in an equilibrium of aggregates [37,43] in which PEP drives the equi-

librium towards the tetramer, while malate shifts it towards the dimer. The absence of cooperativity for PEP binding indicates that if both forms are present in solution, one is inactive or both are active but have the same specificity for PEP. Although different conformational states of the *Csi*PEPCase could be postulated, it is unclear at present whether the reversible association–dissociation processes could be of importance for *in vivo* enzyme regulation and the role that PEP would play in it.

5. Conclusions

A procedure for the successful cloning of *Csi*PEPCase has been described that is suited for the production of milligram quantities of highly active and phosphorylatable enzyme. The purified recombinant enzyme shows properties compatible with those described for other plant-type PEPCases and an unusual high molecular mass structure that is dependent on the presence of PEP. Overall, the kinetic and regulatory properties of *Csi*PEPCase suggest that its role in the fruit could be the production of organic acids in the early stages and the reoxidation of NADH in the environment of reduced respiration of the mature fruit. Further work analyzing in depth the effects of phosphorylation on *Csi*PEPCase will shed light into the mechanism of *in vivo* regulation of this key enzyme of carbon metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2010.08.003.

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