Purification and Properties of Phosphoenolpyruvate Carboxylase from Immature Pods of Chickpea (*Cicer arietinum* L.)

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**ABSTRACT**

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) was purified to homogeneity with about 29% recovery from immature pods of chickpea using ammonium sulfate fractionation, DEAE-cellulose chromatography, and gel filtration through Sephadex G-200. The purified enzyme with molecular weight of about 200,000 daltons was a tetramer of four identical subunits and exhibited maximum activity at pH 8.1. Mg²⁺ ions were specifically required for the enzyme activity. The enzyme showed typical hyperbolic kinetics with phosphoenolpyruvate with a \( K_m \) of 0.74 millimolar, whereas sigmoidal response was observed with increasing concentrations of HCO₃⁻ with \( S_{50} \) value as 7.6 millimolar. The enzyme was activated by inorganic phosphate and phosphate esters like glucose-6-phosphate, \( \alpha \)-glycerophosphate, 3-phosphoglyceric acid, and fructose-1,6-bisphosphate, and inhibited by nucleotide triphosphates, organic acids, and divalent cations Ca²⁺ and Mn²⁺. Oxaloacetate and malate inhibited the enzyme noncompetitively. Glucose-6-phosphate reversed the inhibitory effects of oxaloacetate and malate.

**MATERIALS AND METHODS**

**Chemicals.** All the biochemicals used in the present investigation were procured from Sigma.

**Plant Material.** Immature pods after removing seeds were collected, just before use, from chickpea (*Cicer arietinum* L., cv H-75-35) plants grown in the field of the Department of Agronomy, Haryana Agricultural University, Hisar.

**Enzyme Assay.** Phosphoenolpyruvate carboxylase activity in crude extract or in the fractions eluted from DEAE-cellulose and Sephadex G-200 columns was measured spectrophotometrically at 340 nm. The enzyme activity was coupled to commercial pig heart malate dehydrogenase (EC 1.1.1.37) and the rate of oxidation of NADH was monitored as described by Hatch (8). The standard assay mixture in a final volume of 1.5 ml contained: 5 mM PEP, 50 mM Tris-HCl (pH 8.1), 5 mM MgCl₂, 2 mM DTT, 0.2 mM NADH, 20 mM NaHCO₃, pig heart malate dehydrogenase (2 units), and an appropriate amount of the enzyme preparation. The reaction was initiated by addition of PEP after a 2 min preincubation of the reaction mixture at 30°C. Assays of either crude extract or the purified enzyme were linear with respect to the amount of the enzyme and with time for up to at least 5 min. All assays were performed in duplicate and the average values are reported. The enzyme activity has been expressed as nmol NADH oxidized/min at 340 nm under the specified conditions.

**Protein Estimation.** Protein in various fractions from DEAE-cellulose and Sephadex G-200 columns was monitored by measuring the extinction at 280 nm. Quantitative estimation of protein at each step of purification, however, was done by following the method of Lowry et al. (14).

**Enzyme Purification.** Unless otherwise stated, all steps of enzyme purification were carried out at 0 to 4°C.

Fifty g of fresh immature pods were homogenized in a pre-chilled mortar and pestle using acid washed quartz sand with 50 mM Tris-HCl (pH 8.1) containing 20 mM MgCl₂, 2 mM EDTA, 10 mM 2-mercaptoethanol, 45 mM ascorbic acid, 1% (v/v) PVP, and 20% (v/v) glycerol. The resulting homogenate was passed through four layers of cheese cloth and the filtrate centrifuged at 15,000g for 30 min. The supernatant so obtained was referred to as crude extract. PEP carboxylase in the crude extract was precipitated between 40 and 55% saturation of (NH₄)₂SO₄. The precipitates obtained, after centrifuging at 15,000g for 30 min, were dissolved in 50 mM Tris-HCl buffer (pH 8.1) containing 20 mM MgCl₂, 2 mM EDTA, 2 mM DTT, and 20% (v/v) glycerol (buffer A) and dialyzed against the same buffer for 24 h with at least three changes of buffer.

**DEAE-Cellulose Chromatography.** An aliquot of the dialyzed 40 to 55% (NH₄)₂SO₄ fraction was layered on DEAE-cellulose column (30 x 2.5 cm) previously equilibrated with buffer A. The column was washed with three bed volumes of the above buffer. The enzyme was eluted with buffer B (buffer A containing 0.1 M NaCl) at the flow rate of 30 ml/h and fractions of 4 ml each were collected. The active fractions (approximately 55 ml) eluted as a single peak were pooled. To this solid (NH₄)₂SO₄ was added to 60% saturation. The precipitates collected after centrifugation were dissolved in buffer A and dialyzed against the same buffer.

**Sephadex G-200 Chromatography.** The enzyme fraction obtained from the previous step was loaded onto a Sephadex G-
Table I. Purification of PEP Carboxylase from Immature Pods of Chickpea

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Recovery</th>
<th>Overall Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>4793.8</td>
<td>1621.4</td>
<td>2.9</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>40–55% (NH4)2SO4 precipitate</td>
<td>5562.5</td>
<td>113.9</td>
<td>48.8</td>
<td>116.0</td>
<td>16.8</td>
</tr>
<tr>
<td>di-alyzed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>2825.0</td>
<td>34.4</td>
<td>82.1</td>
<td>58.9</td>
<td>28.3</td>
</tr>
<tr>
<td>60% (NH4)2SO4 precipitate</td>
<td>2562.5</td>
<td>30.4</td>
<td>84.3</td>
<td>53.4</td>
<td>29.0</td>
</tr>
<tr>
<td>di-alyzed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>1381.3</td>
<td>10.1</td>
<td>136.7</td>
<td>28.8</td>
<td>47.1</td>
</tr>
</tbody>
</table>

Fig. 1. Estimation of the mol wt of native PEP carboxylase. A Sephadex G-200 column for gel filtration of the native protein was calibrated with catalase, aldolase, alcohol dehydrogenase, and BSA (in descending mol wt).

Fig. 2. Effect of varying concentrations of PEP on the activity of purified PEP carboxylase, with fixed concentration of HCO₃⁻.

Fig. 3. Effect of varying concentrations of HCO₃⁻ on the activity of purified PEP carboxylase with fixed concentration of PEP.

Fig. 4. Effect of pH on the activity of purified PEP carboxylase.

200 column (60 x 2.0 cm) preequilibrated with buffer B and subsequently eluted with the same buffer. The enzyme was collected in fractions of 2 ml each at a flow rate of 10 ml/h and the active fractions pooled together.

Determination of Purity, Molecular Weight and Molecular Weight of Subunits of the enzyme. The purity of the enzyme preparation obtained from gel filtration through Sephadex G-200 was judged by PAGE at 4°C in 7.5% gel, using Tris-glycine buffer (pH 8.3) following the method of Davis (4). Fifty μg enzyme protein were loaded onto the top of the gel and a current of 3 mamp per gel tube was maintained throughout the process. The mol wt of the purified enzyme was estimated by passing it through a Sephadex G-200 column which had previously been calibrated with catalase (Mₐ 210,000), aldolase (Mₐ 158,000), alcohol dehydrogenase (Mₐ 125,000), and BSA (Mₐ 67,000) and also by applying the following equation of Squire (26).
PEP CARBOXYLASE FROM IMMATURE CHICKPEA PODS

Fig. 5. Effect of varying concentrations of \( \text{Mg}^{2+} \) on the activity of purified PEP carboxylase with fixed concentrations of PEP and HCO\(_3\)-.

Fig. 6. Effect of varying concentrations of glucose-6-P (---), \( \alpha \)-glycerophosphate (---), 3-P-glyceric acid (---), fructose-1,6-bisP (---), and Pi (---) on the activity of purified PEP carboxylase.

Fig. 7. Noncompetitive inhibition of PEP carboxylase, purified by gel filtration through Sephadex G-200, at different fixed concentrations of oxaloacetate and varying the concentration of PEP. OAA, oxaloacetate.

Fig. 8. Noncompetitive inhibition of purified PEP carboxylase at various fixed concentrations of malate and varying the concentration of PEP.

\[
M^{\text{eff}} = 151 \left[ 1.47 - \left( \frac{V_o}{V_e} \right)^{0.9} \right]
\]

where

- \( M \) = molecular weight
- \( V_o \) = void volume
- \( V_e \) = elution volume.

Subunit Molecular Weight. The subunit mol wt was determined by SDS-PAGE carried out in 10% gel at 4°C according to the method of Weber and Osborne (30). BSA (\( M_r \), 67,000), egg albumin (\( M_r \), 45,000), trypsinogen (\( M_r \), 24,000), and \( \beta \)-lactoglobulin (\( M_r \), 18,400) were used as reference proteins. The protein bands were detected by staining with Coomassie brilliant blue.

RESULTS

Enzyme Purification. The results of the enzyme purification are given in Table 1. The enzyme was purified to homogeneity as judged by PAGE with 47-fold purification and about 29% recovery using (NH\(_4\))\(_2\)SO\(_4\) fractionation, ion exchange chromatography on DEAE-cellulose and gel filtration through Sephadex G-200. During purification, in the dialyzed 40 to 55% (NH\(_4\))\(_2\)SO\(_4\) fraction, much higher enzyme activity was recovered than that in the crude extract. This was possibly due to removal of some low mol wt compounds which might be interfering with enzyme activity. The specific activity of the final preparation was much less than that reported for PEP carboxylase purified from maize (3, 28) and spinach leaves (17). The purified preparation was stable for 3 d when kept at 0 to 4°C.

Purity of the Enzyme Preparation. The purified enzyme, after Sephadex G-200 chromatography, was homogeneous on PAGE performed with 7.5% gel. The stained protein band coincided with the single peak of PEP carboxylase activity obtained from an identical gel, thus confirming that the stained protein band was of PEP carboxylase only. PAGE of the enzyme preparation also ruled out the presence of isoenzymes. Isoenzymic forms of PEP carboxylase have, however, been reported in leaves of maize (18) and sorghum (29).

Determination of Molecular Weight and Subunit Mol Wt of the Enzyme. The mol wt of purified PEP carboxylase, as determined from gel filtration through Sephadex G-200 was found to be about 200,000 (Fig. 1). An almost identical value was obtained when the mol wt was calculated using the equation proposed by Squire (26). PEP carboxylase from maize and other C\(_3\) plants (28) and spinach leaves (17) has been reported to have much higher mol wt of 400,000 and about 560,000 D, respectively.

SDS-PAGE of the enzyme preparation yielded a single protein...
band, indicating that the enzyme is composed of identical subunits. From its \( R_e \) (mobility relative to the tracking dye) value of 0.7, the estimated mol wt of the subunit was about 50,000 D, indicating that the enzyme from immature chickpea pods is a tetramer composed of four identical subunits. Similarly, the enzyme from maize (28) and spinach (17) has been reported to be a tetramer but with monomer mol wt of 100,000 and about 130,000, respectively.

**Properties of Purified PEP Carboxylase.** Figure 2 presents the response of enzyme to various concentrations of PEP. The enzyme showed typical hyperbolic kinetics with \( K_m \) of 0.74 mM. Similar observations have also been reported for the enzyme from maize (3, 20, 28), sugarcane (7), and spinach (17). The enzyme, however, showed sigmoidal response to increasing concentrations of HCO\(_3\)\(^-\) (Fig. 3), indicating cooperative binding of HCO\(_3\)\(^-\) to the enzyme. The \( S_{0.5} \) value for HCO\(_3\)\(^-\) was 7.6 mM, which is much higher than that reported for the enzyme from maize (28), spinach (17), and CAM plants (21).

The activity of PEP carboxylase was measured over a pH range from 5.0 to 10.0 (Fig. 4). The purified enzyme exhibited optimum activity at pH 8.1. Similarly, the enzyme from maize, spinach, and many other plants (22) has been reported to exhibit maximum activity near pH 8.1.

Heating the enzyme preparation at 45°C for 2 min resulted in 56% loss in the enzyme activity, whereas the enzyme was completely inactive when heated at 60°C for 2 min. Complete loss of enzyme activity was also observed by Bhagwat and Sane (3) when the enzyme preparation from maize leaf was incubated at 70°C for 30 s.

The enzyme showed hyperbolic kinetics in response to increasing Mg\(^{2+}\) concentrations (Fig. 5). Such a hyperbolic response has also been reported for the enzyme from maize (3, 28) and spinach leaves (17). \( K_m \) for Mg\(^{2+}\) as calculated from the double reciprocal plot was 0.56 mM, which is in accordance with the values reported for the enzyme from maize (3).

**Activators and Inhibitors.** Pi and a number of phosphate esters were found to activate the purified enzyme (Fig. 6). At 8 mM, glucose-6-P caused maximum activation of the enzyme followed by α-glycerophosphate, 3-P-glyceric acid, fructose-1,6-bisP, and Pi. However, at 2 mM concentration, Pi caused maximum activation of the enzyme. Similar effects have been described by Wong and Davies (32) while studying PEP carboxylase from etiolated maize seedlings. Glucose-6-P has been reported to be a potent activator of PEP carboxylase from C4 (28) and CAM (21) plants.

None of the amino acids tested at a 5 mM concentration, had any significant effect on the enzyme activity. This is in contrast to the reported 70% stimulation of the enzyme by glycine from maize leaves (20, 28).

Effect of AMP, ADP, ATP, CMP, CDP, CTP, GDP, GTP, UDP, UTP, IMP, and XMP, each at 5 mM concentration, was studied on the enzyme activity. Nucleotide monophosphates and diphosphates did not exert any effect on the enzyme activity. However, the enzyme was inhibited to the extent of about 28, 28, 14, and 10%, respectively, by ATP, UTP, CTP, and GTP. PEP carboxylase from maize (20, 32) has also been shown to be inhibited by ATP. However, AMP, GMP, and CMP have been reported to activate the enzyme from etiolated maize seedlings (32).

The purified enzyme required Mg\(^{2+}\) ions specifically for its activity. The enzyme showed no activity in absence of Mg\(^{2+}\) and also when Mn\(^{2+}\) or Ca\(^{2+}\) were present alone. The enzyme from maize (28) and spinach leaves (17), however, did not show absolute dependence on Mg\(^{2+}\), but its activity was stimulated in the presence of this divalent cation. Mn\(^{2+}\) and Ca\(^{2+}\), when present at 5 mM concentration in the presence of 5 mM Mg\(^{2+}\), inhibited the enzyme by 72 and 83%, respectively. Inhibition of the enzyme activity by Mn\(^{2+}\) and Ca\(^{2+}\) has also been reported for PEP carboxylase from maize (19) and sugarcane (7) plants.

Oxaloacetate, the product of enzyme reaction, inhibited the enzyme activity substantially. 50% inhibition occurring at 0.2 mM concentration. Kinetic analysis indicated that oxaloacetate was a noncompetitive inhibitor of PEP carboxylase (Fig. 7) with respect to PEP (\( K_i = 0.115 \) mM). Similar observations have been made by Lowe and Slack (13) while working on maize leaf PEP carboxylase.

Malate, which is the major product of photosynthesis in malate forming C4 plants, at 12 mM concentration inhibited the enzyme activity by 33%. However, inhibition by malate was not that profound as has been reported for the enzyme from maize (3) and other C4 plants (12). Nishikido and Takashima (20) and Lowe and Slack (13) also failed to observe any significant inhibition of the enzyme activity by malate. In accordance with the earlier reports from maize and other C4 plants (16), malate also inhibited the enzyme noncompetitively with \( K_i \) of 13.8 mM (Fig. 8). However, the inhibition constant has been reported to be about 6 mm (22). Addition of glucose-6-P relieved the inhibition of the enzyme by oxaloacetate and malate. Similar effect has been reported by Nott and Osmond (21). Other organic acids like succinate, fumarate, glycolate, and malonate, when tested at 5 mM concentrations, did not produce any significant effect on the enzyme activity.

**DISCUSSION**

PEP carboxylase has been purified to homogeneity from immature pods of chickpea using (NH\(_4\))\(_2\)SO\(_4\) fractionation, DEAE-cellulose chromatography, and gel filtration through Sephadex G-200. The enzyme is a tetramer, composed of four identical subunits.

The purified enzyme exhibited some properties resembling those from C4 and C3 plants. However, in some other respects, its properties did not conform to either of the two groups of these plants. The high \( S_{0.5} \) value for HCO\(_3\)\(^-\) is commensurate with the physiological conditions prevalent in pods. Since extracellular CO2 cannot readily diffuse out of podwalls and the CO2 concentration inside the pod is very high as compared to its concentration in the atmosphere (1), the high \( S_{0.5} \) value for HCO\(_3\)\(^-\) and sigmoidal response to increasing concentrations of this substrate thus indicate that the functioning of this enzyme is favored under conditions when the respiratory CO2 losses are high, which essentially occur during pod development. PEP carboxylase could therefore, play an important role, by recycling the CO2 released during either dark respiration or photorespiration, in minimizing carbon losses and enhancing plant productivity and thus contribute to the carbon economy of the developing pods. This view is supported by the presence of various enzymes of photosynthetic carbon reduction cycle and C4 dicarboxylic acid cycle including NADP-malate dehydrogenase and NADP malic enzyme in this tissue (HR Singh, IS Sheoran, R Singh, unpublished data). The same has also been advocated in previous studies on different legume fruits (6, 10, 15, 25). However, the extent of CO2 recycled in relation to the total respiratory losses, the origin of PEP, and the fate of recycled CO2 remains to be conclusively proved. Theoretical limitations, however, do exist for the amount of CO2 that could be recycled via PEP carboxylase reaction as PEP is an energetically rich compound.

Legume seeds being rich in proteins require a large supply of amino acids, for the synthesis of which carbon skeletons are derived from tricarboxylic acid cycle. PEP carboxylase by playing an anaplerotic role in replenishing the intermediates of the above cycle might also be helping the synthesis of amino acids in developing seeds.

Oxaloacetate and malate, the sequential products of CO2 fixation by PEP carboxylase, inhibit the enzyme activity. In vivo,
the concentration of oxaloacetate would be considerably less than that of L-malate (27) and also in view of the fact that oxaloacetate rarely seems to accumulate in plant cells, it is likely that L-malate would be relatively more important as a possible allosteric regulator of PEP carboxylase, thus regulating the CO₂ fixation by feedback or end product inhibition of the enzyme. Detailed mechanisms of the inhibition of PEP carboxylase by oxaloacetate and malate and physiological significance of these phenomena are currently under investigation in our laboratory. However, in view of the earlier report by Winter (31), it may be assumed that pH in the cytosol might be controlling the degree of inhibition of this enzyme by malate.

The reversal of inhibitory effects of oxaloacetate and malate by glucose-6-P is interesting. In C₃ plants, PEP for anapleurotic reactions is derived from glycolytic reactions. When sufficient amount of glucose-6-P is available in the cell to ensure continued production of PEP, the inhibitory effects of oxaloacetate and malate are suppressed. It is thus likely that glucose-6-P and oxaloacetate/malate through their opposing effects might be important in controlling PEP carboxylase activity in vivo as proposed by many others.

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