Purification and characterization of cytosolic pyruvate kinase from developing seeds of *Brassica campestris* L.

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Pyruvate kinase (ATP: Pyruvate phosphotransferase, EC 2.7.1.40; PK) is an important regulatory enzyme of the glycolytic pathway, catalysing the irreversible synthesis of pyruvate and ATP from phosphoenolpyruvate (PEP) and ADP. The plant enzyme is now known to exist in two isoenzymic forms designated as cytosolic and plastidic pyruvate kinase (PKc and PKp). These isoforms differ not only in their respective physical and kinetic characteristics but are also immunologically unrelated and genetically distinct\(^1\). Compared to the extensive work done with PK from non-plant sources, relatively little is known regarding the detailed kinetic characteristics of PKc and PKp from plant sources\(^1\). The main reason for having less information on plant enzyme has been the problem of getting purified preparation from these sources. Earlier work on plant PK was, therefore, conducted on the partially purified enzyme preparations. The purification of cytosolic PK to apparent homogeneity for the first time, was reported from the endosperm of germinating castor beans\(^1\). Following this, homogeneous preparations of PK isoenzymes have been obtained from a number of plant sources\(^2\)-\(^14\). The cytosolic isoenzyme is now known to be regulated by changes in the concentration of several metabolites, by cytosolic pH and by the energy status of the cell\(^1\), and is probably involved in supplying carbon skeletons for ammonium assimilation\(^1\). Besides this, PK has also been shown to regulate the partitioning of carbon between starch, respiration and ammonium assimilation in the green algae *S. minutum*\(^17\). In developing seeds of oil crops, cytosolic PK is utilized in different ways including its direct entry into leucoplasts and vacuoles. In leucoplasts, it joins the pool of PEP and is converted into pyruvate through the action of PKp\(^1\). In cytosol, it could be converted to pyruvate via PKc or malate through the concerted actions of PEP carboxylase and cytosolic malate dehydrogenase. Malate thus produced enters the leucoplasts through recently identified malate translocator\(^18\) and is converted to pyruvate through the action of NADP-malic enzyme\(^17\). Pyruvate in the leucoplasts ultimately serves as the source of carbon for fatty acid synthesis via its conversion into acetyl CoA through the action of pyruvate dehydrogenase complex located in the leucoplasts. Accordingly,
partitioning of PEP into different directions as stated above would be determined by the activity of individual enzymes, which in turn will be controlled by various regulatory mechanisms. We have already purified and characterized PEP carboxylase, PEP phosphatase, NADP-malate enzyme, and plastidic pyruvate kinase from the developing seeds of *Brassica campestris*. Here we report the kinetic characteristics of cytosolic pyruvate kinase from the above source.

Materials and Methods

**Plant material**

Immature seeds of *Brassica campestris* L., var. Toria procured from the Department of Plant Breeding of CCS, Haryana Agricultural University, Hisar were used.

**Chemicals**

All biochemicals used were from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were of analytical grade (B.D.H. or E. Merck, India).

**Enzyme assay**

The enzyme was assayed by coupling pyruvate production to NADH oxidation using lactate dehydrogenase (EC 1.1.1.27) and following the change in absorbance at 340 nm. The standard reaction mixture (1 ml) contained the following: HEPES (100 mM; pH 6.8), KCl (50 mM), MgSO₄ (10 mM), NADH (0.1 mM), ADP (1.5 mM), lactate dehydrogenase (4 units), PEP (1.0 mM) and the enzyme preparation (100 μl). The reaction carried out at 30°C was initiated by the addition of PEP. The enzyme activity is expressed as μmol NADH oxidized min⁻¹ at 340 nm under the specified conditions. In all cases, reaction mixtures without ADP were included in order to correct for PEP phosphatase activity. Coupling enzymes were desalted before use. Assays of either crude extract or of the purified enzyme were linear with respect to the amount of the enzyme and with time for up to at least 5 min. While carrying out pyruvate inhibition studies, the enzyme was assayed by coupling the production of ATP to hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and the reduction of NAD⁺ was monitored at 340 nm. The reaction mixture contained: HEPES (100 mM; pH 6.8), MgSO₄ (10 mM), KCl (50 mM), ADP (1.5 mM), PEP (1.0 mM), pyruvate (5 or 10 mM), glucose (10 mM), hexokinase (1 unit), glucose-6-phosphate dehydrogenase (1 unit), NADP (0.2 mM), and enzyme in a final volume of 1 ml. The reaction was initiated by the addition of PEP. For metal ion studies, the enzyme preparation was dialyzed against buffer to make it free of metal ions. Kinetic studies were performed by varying assay conditions as indicated in legends to Figures 4-9.

**Enzyme purification**

Unless otherwise stated, all steps of enzyme purification were carried out at 4°C.

**Preparation of crude extract**

Fresh immature seeds (50 g, collected from pods at 20 days after anthesis) were homogenized in a prechilled pestle and mortar using acid washed quartz sand with Bicine (50 mM; pH 7.6) containing EDTA (1 mM), MgSO₄ (10 mM), 2-mercaptoethanol (5 mM) and ethylene glycol (20%, v/v) (buffer A). Insoluble PVP (2%, w/v) was added to buffer A just before extraction. The resulting homogenate was passed through four layers of cheese cloth and the filtrate centrifuged at 10,000×g for 30 min. The supernatant so obtained was referred to as the crude extract.

**(NH₄)₂SO₄ fractionation**

Pyruvate kinase in the crude extract was precipitated between 20-50% saturation of (NH₄)₂SO₄. The precipitates obtained after centrifugation at 12,000×g for 30 min were resuspended in buffer A and dialyzed overnight against 10 mM Bicine (pH 7.6) containing 1 mM EDTA, 5 mM 2-mercaptoethanol, 10 mM MgSO₄ and 25% (v/v) ethylene glycol (buffer B).

**DEAE-cellulose chromatography**

The dialyzed enzyme preparation was then layered onto DEAE-cellulose column (35×2.6 cm) pre-equilibrated with buffer B. The column was first eluted with buffer B and then with a gradient of 0-0.3 M KCl added to buffer B at a constant flow rate of 40 ml hr⁻¹. Fractions of four ml each were collected and monitored for protein content and enzyme activity. The enzyme got eluted from DEAE-cellulose column as two distinct peaks designated as PKp (eluted with
0.1 M KCl) and PKc (eluted with 0.2 M KCl). The active fractions of two peaks were pooled separately and concentrated by osmosis against solid sucrose.

**Sepharose-CL-6B chromatography**

The concentrated PKc was then loaded onto a Sepharose-CL-6B column (55 x 1.8 cm) previously equilibrated with 25 mM Bicine (pH 7.6) containing 0.5 mM EDTA, 10 mM MgSO₄, 5 mM 2-mercaptoethanol, 25% (v/v) ethylene glycol, and 50 mM KCl (buffer C). The enzyme was eluted with the above buffer at a flow rate of 18 ml hr⁻¹. The most active fractions (1.5 ml each) eluted as a single peak were pooled and concentrated again by osmosis against solid sucrose.

**Affinity chromatography**

The concentrated enzyme was further purified by passing it through a column of reactive Blue Sepharose-CL-6B (8 x 1.8 cm), previously equilibrated with buffer C containing 0-0.2 M KCl at a flow rate of 18 ml hr⁻¹. Fractions of 1 ml each were collected and analysed for protein and enzyme activity. The active fractions were pooled and stored at 4°C for further studies.

**Determination of purity and molecular weight**

The purity of the enzyme preparation obtained from affinity chromatography column was judged by native PAGE at 4°C in 7.5% gel, using Tris-glycine buffer (pH 8.3) following the method of Davis. The molecular mass of the purified enzyme was estimated by passing it through a Sepharose-CL-6B column which had previously been calibrated with bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), apoferritin (443 kDa), and thyroglobulin (669 kDa). The subunit molecular weight was determined by SDS-PAGE according to the procedure followed by Laemmli. The molecular weight markers used were lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), egg albumin (45 kDa), and bovine albumin (66 kDa).

**Results and Discussion**

**Purification**

The results of purification are given in Table 1. The enzyme was purified to apparent homogeneity as judged by native PAGE with 117 fold purification and about 22% recovery using (NH₄)₂SO₄ fractionation, ion exchange chromatography on DEAE-cellulose (Fig. 1), gel filtration through Sepharose CL-6B (Fig. 2) and affinity chromatography through reactive Blue Sepharose CL-6B. Ion exchange chromatography of

![Fig. 1 — Elution profile of pyruvate kinase on DEAE cellulose column.](image)

**Table 1 — Purification of pyruvate kinase from developing seeds of Brassica campestris**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity (μmol min⁻¹)</th>
<th>Total protein (mg)</th>
<th>Sp. activity (μmol min⁻¹ mg⁻¹ protein)</th>
<th>Fold purification</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>38.64</td>
<td>568.0</td>
<td>0.07</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fraction (20-50%)</td>
<td>35.62</td>
<td>152.0</td>
<td>0.23</td>
<td>3</td>
<td>92</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>21.02</td>
<td>36.0</td>
<td>0.58</td>
<td>8</td>
<td>54</td>
</tr>
<tr>
<td>Sepharose-CL-6B</td>
<td>8.81</td>
<td>3.1</td>
<td>2.89</td>
<td>42</td>
<td>23</td>
</tr>
<tr>
<td>Reactive blue</td>
<td>8.33</td>
<td>1.0</td>
<td>7.98</td>
<td>117</td>
<td>22</td>
</tr>
</tbody>
</table>

![Image](image)
the dialyzed (NH₄)₂SO₄ fraction eluted with 0.2 M KCl gradient, yielded two peaks with PK activity. Plant pyruvate kinase is now well known to occur in two isoforms with distinct physical, kinetic and immunological properties. The purified cytosolic fraction was free of activities of PEP carboxylase, PEP phosphatase and adenylate kinase and had specific activity equal to about eight units mg⁻¹ protein.

Stability
The purified enzyme was stable at 4°C or at −15°C for about a month, but was unstable at room temperature. No loss of enzyme activity occurred upon incubation for 15 min at 30°C. However, the activity was reduced to 50% at 40°C and the enzyme was completely inactivated at temperatures above 60°C. The enzyme was labile in the absence of Mg²⁺. Therefore, to stabilize the enzyme, Mg²⁺ and ethylene glycol were always added in the extraction and elution buffers in addition to 2-mercaptoethanol.

Preincubation of inactive enzyme with a high concentration of 2-mercaptoethanol, DTT and DTE, restored its activity, suggesting that the enzyme activity was probably modulated through interconversion between protein disulfides and thiol groups. The purified PK from germinating and developing castor bean endosperm was also found to be stable for several months when stored at −80°C.

Molecular weight and subunit molecular weight
The molecular mass of purified cytosolic PK as determined from gel filtration through Sepharose CL-2B was found to be about 214 kDa. SDS-PAGE of the purified preparation yielded two protein bands with molecular mass of 55 and 57 kDa (Fig. 3), suggesting that the enzyme in the present case was a heterotetramer with two types of subunits. The cytosolic isoform of C₃ plants is known to be homotetramer with a subunit molecular mass of 56 kDa or a heterotetramer with a subunit molecular mass of 56 and 57 kDa. However, the cytosolic isoform of C₄ plants and Brassica napus suspension cells has been shown to be homotetramer with molecular mass of 50 kDa and 55 kDa, respectively. PKc from higher plants is now known to be tissue specific, exhibiting marked differences in their respective physical and kinetic/regulatory properties. The subunit structure of the homotetrameric castor cotyledon PKc is comparable in terms of that reported for the PKc of developing castor endosperm but differs from that of the heterotetrameric PKc of castor leaves and germinated endosperm.

Effect of pH
The enzyme activity when determined by buffering the reaction mixture with Tris-maleate (pH 5.6-6.5), HEPES-NaOH (pH 6.5-7.8) and Tris-HCl (pH 7.8-9.0) at 100 mM each, showed a narrow pH optimum centered around pH 6.8 (data not shown). In this respect, the enzyme resembled PK from castor bean endosperm and pod-walls of chickpea.

Substrate dependence
The enzyme showed a hyperbolic response with
increasing concentrations of either PEP or ADP in otherwise standard assay mixtures. The \( K_{in} \) values for PEP and ADP, as calculated from double reciprocal plots, were 0.10 and 0.11 mM, respectively. These values fall in a range reported earlier for PKc from higher plants\(^4,16,20\). The present enzyme could also utilize UDP or GDP, but not CDP, as alternative nucleotide to ADP, but with lower \( V_{\text{max}} \). At the same time, the enzyme had lower affinity for these nucleotides as compared with that for ADP (\( K_{in} \) value 0.16 and 0.19 mM, respectively for UDP and GDP). Nucleotide specificity studied earlier for the enzyme from a number of higher plants\(^4,8,16,20\) also suggested ADP to be the preferred substrate.

**Effect of metal ions**

The enzyme showed an absolute requirement for a monovalent and a divalent cation, which was satisfied by K\(^+\) and Mg\(^{2+}\), respectively. None of the monovalent cations could replace K\(^+\). Similarly, divalent cations like Ca\(^{2+}\), Mn\(^{2+}\), and Co\(^{2+}\) could not replace Mg\(^{2+}\). Rather Ca\(^{2+}\), Co\(^{2+}\), and Ni\(^{2+}\) inhibited Mg\(^{2+}\) dependent activity. The absolute requirement for a monovalent cation K\(^+\) and a divalent cation Mg\(^{2+}\) or Mn\(^{2+}\) for maximal activity has also been demonstrated for the enzyme from other plant sources\(^4,5,8,16,20\). Like the *Brassica* enzyme, the enzyme from chickpea podwall\(^15\) and castor bean endosperm\(^4,5,8\) also exhibited greater activity with Mg\(^{2+}\) than with Mn\(^{2+}\).

**Effect of metabolites**

A number of metabolites were tested as possible effectors of the enzyme at saturating concentrations of substrates. None of the metabolites such as aspartate, malate, alanine, glycine, 2-oxoglutarate, methionine, tryptophan, ascorbate, glutathione (oxidized and reduced), glucose-1-P, 3-PGA and fructose-6-P, at 5 mM each, had little (less than 10\%) or no effect on the activity of the enzyme. However, the enzyme activity was enhanced (>20\%) by glucose-6-P, fructose-1,6-P\(_2\), and inorganic phosphate and inhibited by glutamate, glutamine, fumarate, citrate, isocitrate, oxalate and 2-PGA (Table 2). PKc from mungbean\(^1\) and germinating *Ricinus communis* cotyledons\(^8\) is also known to be inhibited by glutamate, glutamine, 2-PGA, fumarate, citrate, isocitrate and oxalate. Oxalate inhibition, which is believed to be due to close structural similarity between oxalate and the enolate form of pyruvate\(^20\) is known to be the strongest in each case. The TCA cycle intermediates (citrate, isocitrate, 2-oxoglutarate, succinate and/or malate) are also known to be the effective feedback inhibitors of many plant PKcs\(^15,27,28\). Glutamate has also been shown to be a potent allosteric inhibitor of plant PKcs\(^27,28\). These inhibitors may provide a tight feedback control which could closely balance the overall activity of the enzyme with the production of carbon skeletons, required for \( \text{NH}_3 \) assimilation, respiration and other biosynthetic activities in actively metabolizing tissues like developing seeds. The enzyme activity was increased slightly (10-15%) by AMP, GMP and GDP at a concentration of 5 mM each, and inhibited by ATP, GTP and UTP. The maximum inhibition was observed with ATP and was of competitive type with respect to PEP and noncompetitive with respect to ADP. This inhibition by ATP was not due to chelation of Mg\(^{2+}\), since the inhibition was not relieved on increasing Mg\(^{2+}\) concentration up to 30 mM. Ki value with respect to PEP as determined from Dixon's plot was found to be 0.85 mM. Similarly, oxalate inhibition was of competitive type with respect to PEP and noncompetitive with respect to ADP, with Ki value with respect to PEP as 0.45 mM. Similar results were obtained for the enzyme from chickpea podwall\(^15\), mungbean\(^1\) and a number of other plant sources\(^1,29\). As suggested by Ambasht et al\(^1\), the rather high

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Enzyme activity (% of control)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2 mM</td>
</tr>
<tr>
<td>Glutamate</td>
<td>66</td>
</tr>
<tr>
<td>Glutamine</td>
<td>72</td>
</tr>
<tr>
<td>Aspartate</td>
<td>94</td>
</tr>
<tr>
<td>Malate</td>
<td>100</td>
</tr>
<tr>
<td>Alanine</td>
<td>108</td>
</tr>
<tr>
<td>Fru-6-P</td>
<td>106</td>
</tr>
<tr>
<td>Fru-1,1-P_2</td>
<td>106</td>
</tr>
<tr>
<td>Glu-6-P</td>
<td>117</td>
</tr>
<tr>
<td>Glu-1-P</td>
<td>100</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>107</td>
</tr>
<tr>
<td>Glycine</td>
<td>100</td>
</tr>
<tr>
<td>Methionine</td>
<td>111</td>
</tr>
<tr>
<td>3-PGA</td>
<td>106</td>
</tr>
<tr>
<td>2-PGA</td>
<td>76</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>110</td>
</tr>
<tr>
<td>Fumarate</td>
<td>75</td>
</tr>
<tr>
<td>Citrate</td>
<td>79</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>79</td>
</tr>
<tr>
<td>ADP-glucose</td>
<td>79</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>107</td>
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<tr>
<td>Glutathione (ox.)</td>
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<td>Glutathione (red.)</td>
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<tr>
<td>Oxalate</td>
<td>32</td>
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<tr>
<td>Pi</td>
<td>128</td>
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</table>

The concentrations not tested are indicated by dash.
concentrations (5 mM) of metabolites used in all these studies are not likely to be found under physiological conditions. Accordingly, the effect will be even weaker at physiological concentrations. Therefore, the intermediates may not have much regulatory influence individually. However, in combination, they may exert strong inhibitory effect as also observed for mungbean enzyme and may be synergistic in their action.

Initial velocity studies

The enzyme activity was determined at three fixed concentrations of one of the substrates, while the concentration of the other substrate was varied. Double reciprocal plots of varying concentration of PEP versus velocity at three fixed concentrations of ADP (Fig. 4) and vice-versa (Fig. 5) gave a set of lines which intersected to the left of the ordinate, indicating the kinetic mechanism to be sequential, which is in agreement with that reported earlier for the enzyme from a number of plant sources.

Product inhibition studies

These studies were carried out to ascertain whether the binding of the substrates to the enzyme was random or ordered. ATP inhibited the forward reaction competitively with respect to PEP at the fixed saturating (Fig. 6), or subsaturating concentrations of ADP, indicating the forward reaction to be either random or ordered, where PEP binds to the enzyme first and ATP is the last product released from the enzyme. With respect to ADP, ATP inhibited the forward reaction non-competitively both at saturating (Fig. 7) and subsaturating concentrations of PEP. When ADP concentration was varied at the fixed saturating (Fig. 8) and subsaturating (Fig. 9) concentrations of PEP, pyruvate inhibition of the enzyme was mixed and non-competitive, respectively. This would indicate that if the mechanism was
ordered, the first product released would be pyruvate. When PEP was the varied substrate, pyruvate inhibited the enzyme competitively both at saturating and subsaturating concentrations of ADP. As suggested earlier, the inhibition should have been uncompetitive for an ordered and non-competitive for random sequential mechanism. Since pyruvate had to be used at a very high concentration, it might be acting as an analogue of PEP and competing for the free enzyme, forming dead-end complex. This would give competitive inhibition. The substrate interaction and product inhibition data except for pyruvate inhibition presented here are consistent with a compulsory ordered Tri-Bi mechanism as suggested earlier by Podesta and Plaxton.

The cytosolic PK from developing seeds of Brassica is probably regulated through the energy status of the cell and intermediates of the TCA cycle. This is supported by the fact that the activity of the plant PK varies with changes in the rates of mitochondrial respiration. Moreover, activation of Brassica enzyme by fructose-1,6-P₂ also supports the role of this enzyme in respiratory control of the cell. However, significant AMP activation or ATP inhibition occurred only at excessive, non-physiological concentrations of these compounds and the $K_m$ for ADP was higher than the level found in vivo. The in vivo regulation of PKc by energy charge is probably very little. This is true with PKcs from a large number of other plant sources as well. Accordingly, ATP and AMP, except in anoxia tolerant tissues, are usually not considered to be critical effectors of plant PKc. The TCA cycle intermediates also seen in the present case are effective inhibitors of plant PKcs and PEP carboxylase. Glycolytic flux will rise as and when TCA cycle intermediates are consumed via anabolism or respiration. PEP carboxylases studied so far are inhibited by malate. This inhibition is relieved by the activator glucose-6-P. Brassica seeds being rich in protein also, require a large supply of carbon

Fig. 7—Lineweaver-Burk plot of product inhibition of PKc by ATP at saturating concentration of PEP with ADP as varied substrate [The reaction mixture contained ATP (o), nil; (▲), 2 mM and (■), 6 mM, respectively].

Fig. 8—Lineweaver-Burk plot of product inhibition of PKc by pyruvate at saturating concentration of PEP with ADP as varied substrate [The reaction mixture contained pyruvate (o), nil; (▲), 1 mM and (■), 2 mM, respectively].

Fig. 9—Lineweaver-Burk plot of product inhibition of PKc by pyruvate at subsaturating concentration of PEP with ADP as varied substrate [The reaction mixture contained pyruvate (o), nil; (▲), 10 mM and (■), 20 mM, respectively].

Fig. 10—Lineweaver-Burk plot of product inhibition of PKc by pyruvate at subsaturating concentration of PEP with ADP as varied substrate [The reaction mixture contained pyruvate (o), nil; (▲), 10 mM and (■), 20 mM, respectively].

Fig. 11—Lineweaver-Burk plot of product inhibition of PKc by pyruvate at subsaturating concentration of PEP with ADP as varied substrate [The reaction mixture contained pyruvate (o), nil; (▲), 10 mM and (■), 20 mM, respectively].

Fig. 12—Lineweaver-Burk plot of product inhibition of PKc by pyruvate at subsaturating concentration of PEP with ADP as varied substrate [The reaction mixture contained pyruvate (o), nil; (▲), 10 mM and (■), 20 mM, respectively].

Fig. 13—Lineweaver-Burk plot of product inhibition of PKc by pyruvate at subsaturating concentration of PEP with ADP as varied substrate [The reaction mixture contained pyruvate (o), nil; (▲), 10 mM and (■), 20 mM, respectively].
skeletons for amino acid biosynthesis. The fact that the *Brassica* enzyme is inhibited by glutamate suggests that this enzyme may also be regulated by the cellular need for carbon skeletons required for amino acid biosynthesis as well.

References