Isolation and Characterization of Phosphoenolpyruvate Phosphatase from Germinating Mung Beans (Vigna radiata)\(^1\)

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ABSTRACT

A phosphoenolpyruvate (PEP) phosphatase was purified to homogeneity from germinating mung beans (Vigna radiata). It was found to be a tetrameric protein (molecular mass 240,000 daltons) made up of apparently identical subunits (subunit molecular mass 60,000 daltons). It was free from bound nucleotides. It did not show pyruvate kinase activity. The enzyme showed high specificity for PEP. Pyrophosphate and some esters (nucleoside di- and triphosphates) were hydrolyzed slowly and phosphoric acid monoesters were not hydrolyzed. The enzyme showed maximum activity at pH 8.5. At this pH, the \(K_m\) of PEP was 0.14 millimolar and the \(V_{max}\) was equal to 1.05 micromoles pyruvate formed per minute per milligram enzyme protein. Dialysis of the enzyme against 10 millimolar triethanolamine buffer (pH 6.5), led to loss of the catalytic activity, which was restored on addition of Mg\(^{2+}\) ions (\(K_m = 0.12\) millimolar). Other divalent metal ions inhibited the Mg\(^{2+}\)-activated enzyme. PEP-phosphatase was inhibited by ATP and several other metabolites.

A PEP\(^1\)-phosphatase activity has been reported to accompany pyruvate kinase in several plant sources (1, 8, 9, 25). It has been referred to as an 'interfering phosphatase,' because of its interference in the pyruvate kinase assay and due to the lack of success in separating the two activities (1, 18, 25). Recently, some reports have been published on the isolation of plant pyruvate kinase from green alga Selenastrum minutum (14) and from germinating castor endosperm (20) which are free from PEP-phosphatase activity. The present paper describes the isolation and characterization of a specific PEP-phosphatase from germinating mung beans. During review of our manuscript, our attention was drawn to the isolation of a PEP-phosphatase from Brassica nigra suspension cells (7).\(^3\)

MATERIALS AND METHODS

PEP tricyclohexylammonium salt was purchased from Boehringer, Mannheim, West Germany. DEAE-cellulose, CM-sephadex, triethanolamine hydrochloride, NADH, amino acids, standard proteins (molecular mass determination and lactate dehydrogenase were from Sigma Chemical Co., St. Louis, MO. Metabolites used for inhibition studies were either from Sigma or Boehringer. Chemicals for gel electrophoresis were from T. Schuchardt, München, West Germany. All other reagents were guaranteed reagent grade chemicals of Sarabhai M. Chemicals, Baroda, India or of E. Merck, India. All solutions were prepared in double distilled water from an all glass (Corning) assembly. Mung beans (Vigna radiata) were the usual edible type procured from the local stores.

Enzyme and Protein Assays

PEP-phosphatase activity was assayed by monitoring the rate of conversion of PEP into pyruvate, which was made to react with NADH in the presence of excess lactate dehydrogenase. The rate of disappearance of NADH was monitored spectrophotometrically. The assay solution contained 1.5 mM PEP, 0.15 mM NADH, 2 mM MgCl\(_2\), 5 enzyme units of lactate dehydrogenase, and an aliquot of PEP phosphatase in 50 mM triethanolamine buffer (pH 8.5) at 30°C (test volume 1.0 mL). PEP-phosphatase was added last and absorbance at 366 nm noted every 10 s for 2 min on an Eppendorf photometer. Initial rate of reaction was obtained graphically. \(\epsilon\) NADH at 366 nm = 3.11 \(\times\) 10\(^4\) M\(^{-1}\) cm\(^{-1}\). An enzyme unit has been defined as that amount of enzyme which brings about the disappearance of 1 \(\mu\)mol of NADH in 1 min under test conditions.

For pyruvate kinase assays, the test solution contained 1.0 mM ADP in addition to the above constituents. Difference between the rate of reaction observed in the presence of ADP and that obtained in its absence was taken to represent the pyruvate kinase activity.

For substrates other than PEP, the enzyme activity was assayed by estimating the concentration of Pi released. Substrate (2.0 mM), MgCl\(_2\) (2.0 mM), and appropriately diluted enzyme were incubated in 50 mM triethanolamine buffer (pH 8.5), at 30°C (test volume 1.0 mL) for 1 min after which the reaction was stopped by adding trichloroacetic acid solution (0.2 mL 10% [w/v]). The reaction mixture was centrifuged, if necessary, and an aliquot of the clear solution taken for Pi assay according to Chen et al. (3). A control experiment was run in each case without the enzyme and the appropriate correction applied.

In some experiments with PEP also, the phosphatase activity was assayed by monitoring the phosphate ions formed as
described. These results were compared with those obtained by monitoring the concentration of pyruvate formed under identical conditions.

Protein concentration was determined by the method of Lowry et al. (15) using bovine serum albumin as the protein standard.

**Determination of Molecular Mass of the Enzyme**

A short column (1.0 × 3.5 cm diameter) of Sephadex G-200-120 (preequilibrated with 10 mM sodium phosphate buffer [pH 6.5]) was prepared by gentle suction as described earlier (10, 16). The column was calibrated with standard proteins (Sigma) of known molecular mass. A solution of known concentration of the enzyme (or the standard protein) (1 mL containing about 1 mg protein) was applied and allowed to percolate into the column. The column filtrate was collected by gentle suction and assayed for enzyme/protein. Dilution factor was calculated

\[
\text{Dilution factor} = \frac{\text{Protein concentration in the applied solution}}{\text{Protein concentration in the column filtrate}}
\]

The molecular mass of the PEP-phosphatase corresponding to its dilution factor was read off the calibration curve.

**Polyacrylamide Gel Electrophoresis**

Gel electrophoresis in the absence (23) and presence of SDS (26) were performed at pH 8.3 and 7.0, respectively, as described earlier. Gels were stained with Amido Schwarz or Coomassie blue. For calibration, standard proteins (Sigma) were used.

**Isolation of PEP-Phosphatase**

The enzyme was routinely isolated from healthy 36 h germinated mung beans (rejecting the ungerminated seeds) by the following steps, which were carried out at 0 to 4°C, unless stated otherwise.

**Extraction**

Germinated seeds (150 g wet weight), washed with cold, double distilled water and blotted dry, were homogenized in the presence of chilled extraction buffer (10 mM phosphate buffer [pH 8.0] containing 5 mM β-mercaptoethanol, 150 mM) with 2 to 3 'bursts' of 30 s each at slow speed followed by one 'burst' of high speed in a kitchen blender. The resultant homogenate was squeezed through a double layer of prewashed muslin cloth and a clear extract (140 mL) was collected by centrifugation.

**Heat Treatment**

The extract, divided into several smaller batches (45–50 mL each), was quickly brought to 50°C in a heated water bath with constant shaking. After 25 min, the enzyme solution was chilled rapidly. Precipitated material was removed by centrifugation and the clear supernatant collected (136 mL).

**pH Changes**

The pH of the above solution (6.5) was brought to 5.5 with 0.1 N HCl (added dropwise with stirring). After allowing to stand for 5 min, the suspension was centrifuged to remove the precipitate and pH of the clear supernatant (125 mL) was readjusted to 6.5 with a dilute ammonia solution.

**Ammonium Sulfate Fractionation**

Solid ammonium sulfate was added with stirring to 30% (saturation). The pH was maintained at 6.5. The resultant suspension was stirred for 0.5 h and centrifuged. The pellet was discarded and the supernatant brought to 45% (saturation) with solid ammonium sulfate. After 1 h of stirring, the suspension was centrifuged, the supernatant discarded and the precipitate suspended in a small volume of 10 mM phosphate buffer (pH 6.5), containing 2 mM MgCl₂. This solution was dialyzed against several changes (hourly) of 500 mL each of 10 mM phosphate buffer (pH 6.5), containing 2 mM MgCl₂, to remove ammonium ions (tested with Nessler's reagent). The dialyzed solution was centrifuged again to remove any precipitated material and the clear solution (6.2 mL) collected.

**DEAE-Cellulose Chromatography**

The above solution was applied to a DEAE-cellulose column (2.5 × 25 cm), prewashed according to Peterson and Sober (19) and equilibrated with 10 mM phosphate buffer (pH 6.5). The column was successively washed with the same buffer (150–200 mL) and 0.1 M KCl in the buffer (60–100 mL). In each case, the washing was continued till the eluate was free of proteins (trichloroacetic acid precipitable materials).

PEP-phosphatase was eluted with 0.2 M KCl in the buffer (fraction volume 5–7 mL). Flow rate was approximately 0.5 mL/min throughout. The enzyme activity was eluted in a single peak with 0.2 M KCl solution. The enzymically active fractions were pooled. The enzyme protein precipitated at 95% ammonium sulfate saturation (stirred for 1 h) was collected by centrifugation and dissolved in a small volume of 10 mM phosphate buffer (pH 6.5), containing 2 mM MgCl₂. The dialyzed sample was centrifuged to collect a clear solution (3.3 mL).

**CM-Sephadex Treatment**

The above solution was added to CM-Sephadex, prewashed (19) and equilibrated with 10 mM triethanolamine solution (pH 6.5) (2.5 g CM-Sephadex/10 mg protein) stirred for 1 h and centrifuged. The supernatant was collected. The enzyme was precipitated at 95% ammonium sulfate saturation, dissolved in 10 mM triethanolamine solution (pH 6.5), containing 2 mM Mg²⁺ and dialyzed for 6 h against the same buffer. Clear dialysate (1 mL) was collected by centrifugation.

**RESULTS**

**Purification of PEP-Phosphatase**

PEP-phosphatase activity is low in ungerminated mung beans seeds. It increases on germination, achieves a maximum
value at 36 h of germination, and then declines if the seeds are allowed to germinate for longer periods (data not shown). Similar variations were also observed with pyruvate kinase activity. Both activities were extracted together. However, their relative concentrations vary with pH of the extraction buffer. More of the kinase activity was extracted at higher pH.

A sample protocol of the purification of PEP-phosphatase from 36 h germinated mung beans is shown in Table I. The first two steps (heat treatment and pH changes) do not bring about any significant increase in specific activity but help by making the subsequent steps more effective. Further, the pH change step completely removes the pyruvate kinase activity. Elution profile of the enzyme from the DEAE-cellulose column is shown in Figure 1. PEP-phosphatase has been purified about 60-fold with about 22% recovery. The purified enzyme does not show any pyruvate kinase activity on the addition of ADP (or any other nucleoside diphosphate) to the activity assay solution. The purified enzyme is quite stable when stored frozen at −10°C (half-life about 50 d). The enzyme is relatively more stable at lower pH values. Its half-life (t1/2) at 50°C was found to be 6.8, 2.4, and 1.8 min at pH 6.5, 7.5, and 8.6, respectively. The purified enzyme gives a single protein band following PAGE in the absence (Fig. 2A) as well as presence of SDS (Fig. 2B). It shows a typical protein spectrum, with \( \lambda_{\text{max}} \) at 278 nm and A280/A260 ratio equal to 1.38. Its \( E^{1%}_{280\text{nm}} \) was found to be 0.99.

**Native Molecular Mass and Subunit Molecular Mass**

Using a short Sephadex G-200-120 column (1.0 × 3.5 cm diameter), dilution factors were calculated for several known proteins as explained under "Materials and Methods." The dilution factors were found to be related to the molecular mass of the proteins (Fig. 3) in a similar manner as reported earlier for elution volumes from longer columns. The dilution factor of PEP-phosphatase was found to be equal to 4.1, which corresponds to a molecular mass of 240,000 D (Fig. 3). The dilution factor was not affected by the presence of PEP or other components of the assay solution, suggesting that this enzyme does not undergo any change in its molecular mass in the activity assay solution.

The subunit molecular mass of PEP-phosphatase has been determined by comparing its electrophoretic mobility in the presence of SDS with mobilities of known proteins under identical conditions (Fig. 4). The subunit molecular mass was found to be 60,000 D. Presence of a single protein staining band following SDS-PAGE suggests that the enzyme is made up of subunits of equal molecular mass. Thus, mung bean PEP-phosphatase appears to be a homotetrameric protein.

**Specificity**

Several compounds were tested as substrates and the results are shown in Table II, PPI, ATP, and some nucleoside diphosphates are hydrolyzed at considerably lower rates (10–20%) than that shown with PEP. The monophosphate esters including pyridine were either not hydrolyzed or showed very small rates (less than 5% of that of PEP). Thus, this enzyme is quite specific for PEP and some energy-rich phosphate compounds.

With PEP as substrate, the concentrations of pyruvate and Pi released during enzymic reaction under identical conditions were found to be equal. Further, the pyruvate/Pi ratio remained close to unity (1.0–1.05) when ADP or any other nucleoside diphosphate was present in the reaction solution (during incubation with the enzyme). These results confirmed that PEP was undergoing hydrolysis only and that the enzyme preparation was free from pyruvate kinase activity.

**Steady-State Kinetics**

Variation of rate of hydrolysis at different PEP concentrations obeys simple Michaelis-Menten relationship (linear double reciprocal plot, Fig. 5). The \( K_m \) for PEP was 0.14 mm, and the \( V_{\text{max}} \) was 1.05 μmol pyruvate formed per min per mg enzyme protein at pH 8.5 at 30°C.

The enzyme showed optimal activity at pH 8.5 (Fig. 6). Half-maximal activity was observed at pH 7.2 and 9.8.

Temperature dependence of the rate of enzymic hydrolysis of PEP in the range 20 to 50°C obeys Arrhenius relationship (data not shown) with energy of activation for the catalyzed reaction equal to 10.31 kcal/mol enzyme site.

**Role of Metal Ions**

PEP-phosphatase activity assay solution routinely contained 2 mm Mg²⁺ ions. In the absence of these ions, lower activities (about half the rate) were recorded. When a solution of purified enzyme was dialyzed against several changes of 10 mm triethanolamine buffer (pH 6.5), at 0°C for 48 h, the dialyzed protein did not show any enzyme activity when assayed in the absence of Mg²⁺ ions. Full activity was regained on the addition of Mg²⁺ ions in the assay solution (data not shown). None of the other metal ions tested (Mn²⁺, Hg²⁺, Zn²⁺, Cu²⁺, Ni²⁺) could restore enzyme activity. These results

| Table I. Purification of PEP-Phosphatase from Germinated Mung Beans (150 g Wet Weight) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Steps           | Volume (mL)     | Total Activity  | Specific Activity | Purification (fold) | Recovery (%)   |
|                 |                 | Total Protein (mg) |                 |                  |                 |
|                 |                 |                 | units/mg |                 |                  |                 |
| Extract         | 140             | 51.7            | 2940     | 0.018            |                 | 90.1            |
| Heat treatment  | 136             | 46.6            | 1877     | 0.025            | 1.4             |                 |
| pH treatment    | 125             | 40.2            | 877      | 0.045            | 2.5             | 77.8            |
| Ammonium sulfate fraction (30–45% saturation) | 6.2 | 32.2 | 207 | 0.16 | 8.9 | 62.5 |
| DEAE-cellulose chromatography | 3.3 | 14.0 | 19.8 | 0.71 | 40.3 | 27.1 |
| CM-Sephadex treatment | 1.0 | 11.6 | 11.0 | 1.05 | 58.0 | 22.4 |
show that PEP-phosphatase has an absolute requirement for 
Mg$^{2+}$ ions. Mg$^{2+}$ concentration-dependence of the enzyme
activity of the dialyzed enzyme shows a $K_m$ for Mg$^{2+}$ of
0.12 mM (Fig. 7).

When other metal ions, which fail to restore the activity of
the dialyzed enzyme, are added to the usual activity assay
solution (containing Mg$^{2+}$) they are found to be inhibitory.
The inhibition shows the sequence Hg$^{2+}$ > Zn$^{2+}$ > Cu$^{2+}$ >
Ni$^{2+}$. The inhibition by these ions is found to be competitive
with respect to Mg$^{2+}$ ions (Fig. 8A) and noncompetitive with
respect to PEP (Fig. 8B). Only representative results with Zn$^{2+}$
are shown in Figure 8. The $K_i$ (competitive) values of Cu$^{2+}$ and
Zn$^{2+}$ ions are 0.22 and 0.023 mM, respectively. The corre-
sponding $K_i$ (noncompetitive) (0.62 and 0.10 mM, respectively)
are somewhat higher, as is to be expected because of the high
concentration of the competing Mg$^{2+}$ ions in these experi-
ments.

**Effect of Metabolites**

Some metabolites of the glycolytic pathway, Krebs' tricar-
boxylic acid cycle and glyoxalate pathway have been exam-
dered for their effect on PEP-phosphatase activity. No activa-
tors were found. 3-Phosphoglycerate, citrate, succinate, ox-
alate, and ATP inhibited the enzyme. The inhibition was
competitive in each case. The $K_i$ values are shown in Table
III. The $K_i$ values are not affected by ADP (1.0 mM). If ATP
(5 mM, 43% inhibition) and citrate (5 mM, 46% inhibition)
are taken together, the enzyme is inhibited 73%, suggesting
that inhibitory effects of these two are additive. The following
compounds were also tested and found to have no effect on
the rate of enzymatic hydrolysis of PEP: $\alpha$-ketoglutarate,
malate, l-alanine, or l-phenylalanine (10 mM each); glucose,
glucose-6-phosphate, fructose-1,6-diphosphate, 6-phosphogluco-
conate, aspartate, glutamate, 2-phosphoglycerate, pyro-
phosphate, or AMP (5 mM each); potassium laurate or various
nucleoside diphosphates (1 mM each). Free phosphate ions (5

**Figure 1.** Elution profile of mung bean PEP-phosphatase on DEAE-
cellulose chromatography. (A), enzyme activity units; (O), protein in
mg. The enzyme was eluted with 10 mM potassium phosphate buffer,
(pH 6.5) containing 0.2 M KCl as shown.

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phosphate, or AMP (5 mM each); potassium laurate or various
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**Figure 2.** Polyacrylamide gel electrophoresis of purified mung bean
PEP-phosphatase. A, 50 $\mu$g protein applied at pH 8.3 according to
Reisfeld et al. (23); B, 10 $\mu$g of protein applied in the presence of 1%
(w/v) SDS at pH 7.0 according to Weber and Osborn (26).
Table II. Substrate Specificity of PEP-Phosphatase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity Relative to PEP</th>
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</thead>
<tbody>
<tr>
<td>PEP</td>
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<tr>
<td>PPI</td>
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<td>10</td>
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</tr>
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<td>UDP</td>
<td>8</td>
</tr>
<tr>
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<tr>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>2-P-glycerate</td>
<td>0</td>
</tr>
<tr>
<td>Phytate</td>
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</table>

DISCUSSION

Earlier reports showed that a PEP-phosphatase activity was associated with pyruvate kinase activity of several plants (1, 8, 9, 18, 19). In the present study also, PEP-phosphatase and pyruvate kinase activities were extracted together but the ratio of the two activities changed with pH of the extraction buffer (keeping the pH of the assay buffer the same). This suggests that the two activities may reside on different proteins. This is confirmed by the recent reports on isolation of a PEP-phosphatase (this work and ref. 7) and pyruvate kinase (14, 20) from various plant sources free from the other activity.

Mung bean PEP-phosphatase, purified to homogeneity, has been found to be a tetrameric protein of molecular mass 240,000 D and subunit molecular mass of apparently identical subunits equal to 60,000 D. These values are similar to those of catalytic pyruvate kinase from various sources (4, 5, 11, 12, 14).

Mung bean PEP-phosphatase shows a high specificity for PEP. PPI and some energy-rich pyrophosphate compounds (nucleoside di- and tri-phosphates) were hydrolyzed at greatly reduced rates. Monophosphates were mostly resistant to hydrolysis by this enzyme. The pH optimum of the enzyme was found to be 8.5, which will classify it as an alkaline phosphatase.

Dialysis of a solution of purified PEP-phosphatase against buffer (pH 6.5) led to complete loss of catalytic activity. Full activity was restored on the addition of Mg²⁺ ions. Other metal ions (Mn²⁺, Cu²⁺, Zn²⁺, and Ni²⁺) did not restore the
activity. This shows that Mg$^{2+}$ is essential for the PEP-phosphatase activity.

Other metal ions were found to inhibit the enzyme. The inhibition is competitive with respect to Mg$^{2+}$ and noncompetitive with respect to PEP. Thus, the metal ion inhibition may be attributed to their displacing Mg$^{2+}$ ions from the enzyme site. It is interesting that this displacement does not affect $K_m$ for PEP (noncompetitive inhibition). This suggests that either Mg$^{2+}$ ions are not involved in the binding of the substrate to the enzyme or other metal ions can be equally effective in this role. However, Mg$^{2+}$ must have a specific role to play in the catalytic step because other metal ions cannot substitute for it.

Several metabolites (ATP, 3-P-glycerate, citrate, and succinate) were found to inhibit PEP-phosphatase. The inhibition is competitive with respect to PEP and $K_i$ values lie in a narrow range, namely 3 to 6 mM. These values are higher than the normal physiological concentrations of these compounds (which may approach mM values but are generally somewhat lower). Thus, if the metabolites are considered individually this inhibition will appear to have no physiological relevance. However, the results described here show that the inhibitory effects of these metabolites are additive. Note that sum of the concentrations of these metabolites will approach (or may even exceed) a mean $K_i$ value for the various metabolites (4.40 ± 2.0 mM). Thus, the PEP-phosphatase activity in the cell may be strongly regulated by a combined effect of these (and possibly other) metabolites.

The inhibitory metabolites bind Mg$^{2+}$ ions with different
affinities. This binding may be a contributory factor but does not appear to be the only factor responsible for their inhibitory action, because the sequence of their inhibitory action (oxalate \( \approx \) 3-phosphoglycerate > citrate = ATP > succinate) is different from the sequence of their affinity for Mg\(^{2+}\) ions (ATP > citrate > oxalate > 3-phosphoglycerate > succinate) (2). Mung bean PEP-phosphatase shows very significant differences when compared with the corresponding enzyme recently purified from Brassica nigra suspension cells (7). The latter enzyme was found to be a monomeric protein (molecular mass 56,000 D as compared to tetrameric mung bean enzyme), more thermostable (no loss of activity in 4 min at 50°C cf. 1.8–6.8 min for the mung bean enzyme), showed a broad pH optimum at about pH 5.6 (pH 8.5 for the mung bean enzyme), had a much higher \( V_{\text{max}} \) and a broader substrate specificity, and was activated by several cations (other than Mg\(^{2+}\)) which inhibit the mung bean enzyme. Properties of the B. nigra enzyme are typical of an ‘acid phosphatase’ with a preference for PEP as a substrate. Differences between the two enzymes extend also to the effect of various metabolites and amino acids on their activities. For example, glutamate, aspartate, glucose-6-P and fructose-1,6-P\(_2\) inhibit the B. nigra enzyme, but have no effect on the activity of mung bean PEP-phosphatase.

Several phosphatases have been reported to occur in animal, plant and microbial systems. A review of literature has been carried out specially with respect to their substrate specificity (6, 13, 17, 21, 22). Other than B. nigra PEP-phosphatase (7), none of them exhibits the high degree of specificity for PEP shown by the present enzyme. As described in the preceding paragraph, the B. nigra enzyme exhibits significant differences from the mung bean enzyme. Thus, it appears that the present work deals with an enzyme which has not been characterized earlier. The turnover number of this enzyme for PEP, which is its best known substrate, is quite small. Therefore, a possibility exists that the real physiological substrate of this enzyme may not be PEP.

The function of a PEP-phosphatase in the cellular economy is not clear. This will depend on subcellular localization of this enzyme for which no data is available at present. Recently, it has been suggested that this enzyme may provide an alternate route from PEP to pyruvate (24). This may become significant under conditions of phosphate starvation (7). It is apparent from the above discussion of properties of the mung bean enzyme that under the normal physiological conditions its activity will be strongly inhibited by two factors. First the combined effect of several metabolites mentioned above will inhibit the enzyme considerably. Second, its rather low affinity for Mg\(^{2+}\) \((K_a = 0.12 \text{ mm})\), which is bound to several other cellular constituents, will keep it constantly starved of this essential cation. The advantage which accrues to the cell from inhibition of PEP-phosphatase is obvious, because each PEP molecule is worth one ATP molecule if it is acted upon by pyruvate kinase instead of PEP-phosphatase.

PEP-phosphatase may, however, become more important under some abnormal conditions. If the overall metabolism of the plant is slowed down, \( e.g. \) by fall in the ambient temperature, concentration of various inhibitory metabolites would decrease thus releasing the inhibition of PEP-phosphatase. Under such conditions, PEP may be hydrolyzed by PEP-phosphatase releasing a large amount of energy in the form of heat (a thermogenic function) and making pyruvate available for other metabolite routes.

**LITERATURE CITED**