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Purification and Characterization of a Phospho*enol*pyruvate Phosphatase from *Brassica nigra* Suspension Cells[1]

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ABSTRACT

Phospho*enol*pyruvate phosphatase from *Brassica nigra* leaf petiole suspension cells has been purified 1700-fold to apparent homogeneity and a final specific activity of 380 micromole pyruvate produced per minute per milligram protein. Purification steps included: ammonium sulfate fractionation, S-Sepharose, chelating Sepharose, concanavalin A Sepharose, and Superose 12 chromatography. The native protein was monomeric with a molecular mass of 56 kilodaltons as estimated by analytical gel filtration. The enzyme displayed a broad pH optimum of about pH 5.6 and was relatively heat stable. Western blots of microgram quantities of the final preparation showed no cross-reactivity when probed with rabbit polyclonal antibodies prepared against either castor bean endosperm cytosolic pyruvate kinase, or sorghum leaf phospho*enol*pyruvate carboxylase. The final preparation exhibited a broad substrate selectivity, showing high activity toward *p*-nitrophenyl phosphate, adenosine diphosphate, adenosine triphosphate, gluconate 6-phosphate, and phospho*enol*pyruvate, and moderate activity toward several other organic phosphates. Phospho*enol*pyruvate phosphatase possessed at least a fivefold and sixfold greater affinity and specificity constant, respectively, for phospho*enol*pyruvate (apparent Michaelis constant = 50 micromolar) than for any other nonartificial substrate. The enzyme was activated 1.7-fold by 4 millimolar magnesium, but was strongly inhibited by molybdate, fluoride, zinc, copper, iron, and lead ions, as well as by orthophosphate, ascorbate, glutamate, aspartate, and various organic phosphate compounds. It is postulated that phospho*enol*pyruvate phosphatase functions to bypass the adenosine diphosphate dependent pyruvate kinase reaction during extended periods of orthophosphate starvation.

Acid phosphatases (orthophosphoric-monoester phosphohydrolyases, EC 3.1.3.2) catalyze the hydrolysis of a wide range of orthophosphate monoesters, with a pH optimum of between pH 5.0 to 6.0. Enzymes of this category appear to be ubiquitous, occurring in a broad variety of species and tissues (13). Particulate and soluble plant acid phosphatases are widely distributed and frequently occur as multiple forms which differ in molecular mass, substrate specificity, and electrophoretic mobility (3, 6, 11, 12, 14, 15, 19, 21, 23–25, 29, 31). Environmental stresses such as Pi or water deficiency, or salt excess, apparently cause increases in the acid phosphatase activity of various plant tissues (12, 21).

A special class of acid phosphatases exist which display a clear, but nonabsolute, substrate specificity. For example, plant 3-PGA[2] phosphatase (23, 24, 31) and phytase (11) preferentially utilize 3-PGA and phytic acid, respectively, as their substrate. However, both phosphatases will also hydrolyze Pi from a variety of other phosphorylated substrates. Similarly, nonplant 2-PGA phosphatase (10), and 3 P-glycerol phosphatase (27) (both having nonabsolute substrate specificity) have been reported.

PEP is a key intermediate of plant glycolysis which plays a central role in plant metabolism (8). The presence of a plant phosphatase specific for PEP has been inferred for many years owing to the substantial 'PEP phosphatase' activity which frequently interferes with the determination of plant pyruvate kinase activity (18). PEP phosphatase was recently proposed to provide plants with an alternative metabolic route for the conversion of PEP to pyruvate (26). However, the enzyme has never been purified to allow characterization of its individual biochemical and kinetic properties.

In this paper, we report the purification to apparent homogeneity of a PEP phosphatase from *Brassica nigra* (black mustard) suspension cells and describe physical and kinetic properties of the purified enzyme.

MATERIALS AND METHODS

Chemicals and Plant Material

Biochemicals, cell media ingredients, coupling enzymes, SDS-PAGE molecular mass standards, and bisacrylamide were purchased from Sigma Chemical Company. Tris and SDS were from Schwartz/Mann Biotech. Protein assay reagent was obtained from Bio-Rad. All other reagents were of analytical grade and were obtained from BDH. S-Sepharose, native molecular mass standards, chelating Sepharose 6B, Con A Sepharose 4B, Superose 12 (prep grade), a Superose 12 HR 10/30 column (prepacked), and a FPLC system were obtained from Pharmacia Fine Chemicals. All buffers used in this study were degassed and adjusted to their respective pH values at 25°C.

Heterotrophic *Brassica nigra* suspension cells were cultured from a continuing cell line which had been originally isolated from *B. nigra* leaf petioles. To isolate the original line *B. nigra* seeds were surface-sterilized in 1.8% (w/v) sodium hypochlorite, and 0.02% (w/v) Triton X-100 for 30 min and germi-

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[2] Abbreviations: PGA, phosphoglycerate; PEP, phospho*enol*pyruvate; FPLC, fast protein liquid chromatography; MS, Murashige and Skoog; pNPP, *p*-nitrophenylphosphate.

nated on MS basal medium (20) containing 2% (w/v) sucrose, 1% (w/v) agar and no plant growth regulators. Petioles of the first mature leaves were cut into 2 mm sections and grown to callus on MS agar containing 2,4-dichlorophenylacetic acid (2 mg/L). When calluses reached 5 mm in diameter (at about 3 weeks) they were used to inoculate MS liquid medium containing the same sugar and growth regulator levels (5 calluses per 10 mL liquid MS). Incubation was on a rotational shaker (120 rpm) at 25°C. In 2 weeks this culture was used to initiate a standard 50 mL suspension culture. Subculturing was performed every 7 d by transferring 10 mL of subsequent 1-week-old cultures into 40 mL fresh MS medium. This suspension culture has now been maintained with no apparent loss in vitality for over 2 years.

Large quantities of suspension cells were obtained by increasing the culture volume. Briefly, three 7-d-old 50 mL cultures were used to inoculate single 2-L flasks containing 350 mL MS medium (total volume = 500 mL). After a further 7 d the cells were harvested on a Büchner funnel fitted with a 10 μm nylon filter. The cells (at about 50 g fresh weight per flask) were then washed three times with 100 mL 1 mM $CaCl_2$, frozen in liquid N_2, and stored at −80°C until used.

Enzyme Assays

Assay A

For routine measurements of enzyme activity, the PEP phosphatase reaction was coupled with the lactate dehydrogenase reaction and assayed at 25°C by monitoring NADH utilization at 340 nm using a Varian DMS 200 spectrophotometer. Standard assay conditions for PEP phosphatase were 50 mM sodium acetate (pH 5.6) containing 1 mM PEP, 4 mM $MgCl_2$, 0.20 mM NADH, and 3 units dialyzed rabbit muscle lactate dehydrogenase in a final volume of 1.0 mL. Assays were initiated by the addition of the enzyme preparation. Assays were corrected for NADH oxidase activity by omitting the PEP from the reaction mixture. One unit of enzyme activity is defined as the amount of enzyme resulting in the utilization of 1 μmol NADH/min at 25°C.

Assay B

For substrates other than PEP, the method of Berenblum and Chain (2) was used to detect the Pi released by the phosphatase reaction. Between 0.005 and 0.01 unit of PEP phosphatase was incubated in 3 mL cuvettes with 1 mL of 50 mM sodium acetate (pH 5.6) containing 4 mM $MgCl_2$ and an alternative substrate for 5 min at 25°C. Reactions were terminated by the addition of 2 mL reagent A (reagent A = 0.86 M H_2SO_4 containing 3 mM NH_4Mo and 80 mM ascorbate). Samples were incubated at 45°C for 20 min, and the A_{820} determined. To calculate activities, a standard curve over the range of 0.01 to 0.16 μmol Pi was constructed for each set of assays. Assays were performed in duplicate and controls were run for background amounts of Pi present at each substrate concentration tested, by adding reagent A before the enzyme. Hydrolysis was proportionate to enzyme concentrations of between 0.005 to 0.1 unit per mL incubation mixture and remained linear with time for at least 10 min.

Kinetic Studies

Owing to potent inhibition of the enzyme by Pi, acid washed cuvettes were used for all kinetic studies. Apparent K_m values and Hill coefficients for all substrates and cofactors were calculated from Hill plots. The activation constant (K_a) for Mg^{2+} was determined using a double reciprocal plot of $1/(v\text{-}v_o)$ *versus* $1/[Mg^{2+}]$. Enzyme-inhibitor dissociation constants (K_i) were determined from Dixon plots, and the pattern of inhibition was evaluated by Lineweaver-Burk plots. All kinetic parameters are the means of duplicate determinations performed on two separate preparations of the purified enzyme, and are reproducible to within ±10% SE.

Electrophoresis and Western Blotting

SDS-PAGE was performed using a Bio-Rad mini-gel apparatus and the discontinuous system of Doucet and Trifaró (9). Final acrylamide monomer concentration in the 0.75 mm thick slab gels was 10% (w/v) for the separating gel and 4% (w/v) for the stacking gel. All samples were preincubated in the presence of SDS sample buffer (70 mM Tris-HCl [pH 6.7] containing 8 M urea, 3% [w/v] SDS, 100 mM DTT and 0.005% [w/v] bromophenol blue) for 2 min at 100°C prior to be being loaded on the gels. Gels were run at 160 V constant voltage for 1 h. Following electrophoresis, gels were stained for protein using Coomassie blue R or electroblotted onto nitrocellulose as described elsewhere (16). For the determination of subunit molecular mass using SDS-PAGE, a plot of R_F *versus* log molecular mass was constructed using the following protein standards: myosin (205 kD), β-galactosidase (116 kD), phosphorylase B (97.5 kD), BSA (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD).

Western blots were probed as in Lin *et al.* (16) using either affinity purified rabbit anti-castor bean endosperm cytosolic pyruvate kinase immunoglobulin G (16), or rabbit antiserum (1:200 dilution) prepared against sorghum leaf PEP carboxylase.

Native Molecular Mass Determination

Molecular mass estimations were made on a Superose 12 HR 10/30 column using 0.15 mL sample volumes and 30 mM Mes-HCl (pH 6.0) containing 1 mM DTE, 1 mM EDTA, and 50 mM KCl as column buffer. Fractions (0.25 mL) were collected with a flow rate of 0.2 mL/min and assayed for A_{280} and PEP phosphatase activity. The native molecular mass of PEP phosphatase was determined from a plot of K_{av} (partition coefficient) *versus* log molecular mass for the following protein standards: thyroglobulin (662 kD), appoferritin (443 kD), catalase (232 kD), ADH (150 kD), BSA (66 kD), ovalbumin (45 kD), carbonic anhydrase (29 kD), and ribonuclease (14 kD).

Protein Determination

Protein concentration was measured by the method of Bradford (4) using the Bio-Rad prepared reagent and bovine γ-globulin as standard.

Buffers Used in PEP Phosphatase Purification

Buffer A: 50 mM sodium acetate (pH 5.6) containing 1 mM EDTA, 0.5 mM DTE, 5 mM thiourea, 10 μg/mL leupeptin, 10 μg/mL chymostatin, and 1 mM PMSF; buffer B: 30 mM sodium acetate (pH 4.9) containing 0.25 mM EDTA, and 0.5 mM DTE; buffer C: 25 mM Tris-HCl (pH 7.1) containing 500 mM NaCl; buffer D: 25 mM sodium acetate (pH 4.0) containing 500 mM NaCl; buffer E: 25 mM sodium acetate (pH 4.0) containing 500 mM NaCl, 150 mM NH_4Cl, and 50 mM imidazole; buffer F: 5 mM KPi (pH 6.5) containing 100 mM KCl, 0.5 mM DTE, 1 μM $CaCl_2$, and 1 μM $MnCl_2$; buffer G: 30 mM Mes-HCl (pH 6.0) containing 1 mM DTE, 1 mM EDTA, 50 mM KCl, and 0.03% (w/v) NaN_3.

RESULTS

Purification and Physical Properties of PEP Phosphatase from *B. nigra* Suspension Cells

All purification procedures were carried out at 0 to 5°C unless otherwise specified.

Crude Extract

Quick frozen cells (1 kg) were ground to a powder under liquid N_2. The powder was then thawed in 500 mL buffer A and sonicated for 10 min at maximum power (20 s intervals using a Bronson Sonic Power Co. model W350 Sonifer). The sonicate was centrifuged at 17,000*g* for 20 min. Pellets were reextracted in 300 mL buffer A by grinding with a mortar and pestle containing a small amount of acid washed sand. Reextracted pellets were centrifuged as described above. Supernatants were pooled and designated the crude extract.

Ammonium Sulfate Fractionation

Solid $(NH_4)_2SO_4$ was added with stirring to the crude extract to 35% (saturation). The solution was stirred for 20 min and centrifuged as above. Pellets were discarded and the supernatant adjusted to 80% (saturation) with solid $(NH_4)_2SO_4$. The solution was stirred for 20 min and centrifuged as above. The pellets were resuspended in 150 mL buffer B containing 0.5 mM PMSF, dialyzed overnight against 10 L of the same buffer, and centrifuged at 27,000*g* for 20 min to remove insoluble material.

S-Sepharose Cation Exchange Chromatography

The clear supernatant was absorbed at 2 mL/min onto a column of S-Sepharose (2.5 × 20 cm) which had been preequilibrated in buffer B. The column was connected to a FPLC system, washed with buffer B until the A_{280} decreased to about 0.1, and eluted with a 0 to 0.5 M KCl gradient in buffer B (gradient volume = 450 mL; fraction size = 8 mL). As shown in Figure 1A, a small amount of activity eluted in the buffer B wash. Peak activity fractions were pooled and concentrated overnight against solid PEG (average molecular mass 8 kD).

Chelating Sepharose 6B Chromatography

The concentrated S-Sepharose peak fractions were diluted two-fold with buffer C, adjusted to pH 7.1 with solid Tris,

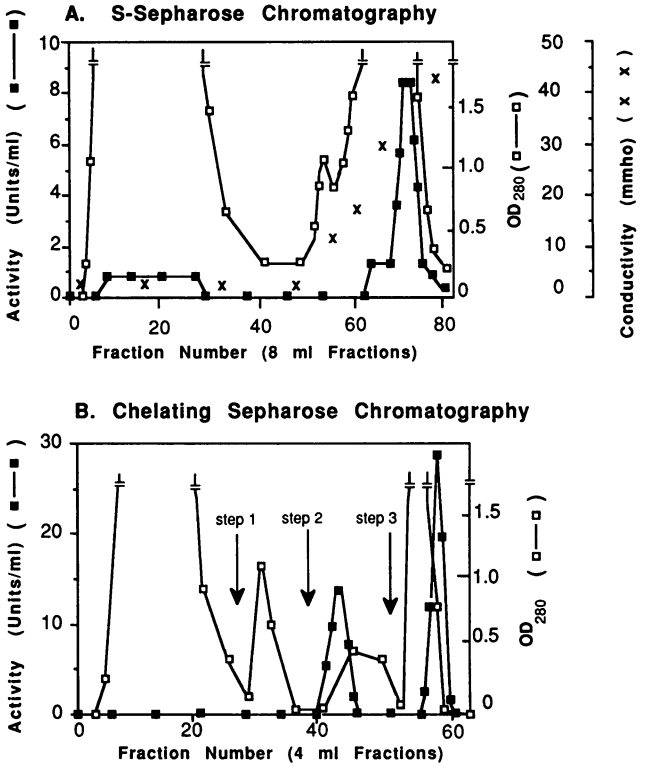

Figure 1. A, S-Sepharose chromatography of acid phosphatase from *B. nigra* suspension cells. Details of the procedure are described in the text. B, Chelating Sepharose chromatography of acid phosphatase from *B. nigra* suspension cells. The enzyme was bound at pH 7.1 and the column sequentially eluted with the following buffers: buffer D (step 1); buffer E (step 2); and buffer E containing 10 mM EDTA (step 3). A_{280} values obtained following elution with buffer E containing 10 mM EDTA are unreliable owing to interference by a Cu^{2+}-EDTA complex which coeluted with phosphatase B activity.

and absorbed at 0.75 mL/min onto a column of chelating Sepharose (1.5 × 10 cm) which had been charged with 18 mM $CuSO_4$, and preequilibrated with buffer C. The column was washed with buffer C and sequentially eluted with buffer D, buffer E, and buffer E containing 10 mM EDTA as shown in Figure 1B. Two peaks of phosphatase activity were eluted (Fig. 1B). The first activity peak eluted with buffer E and was designated phosphatase A, while the second activity peak eluted with buffer E containing 10 mM EDTA and was designated phosphatase B. A blue colored Cu^{2+}-EDTA complex coeluted with the phosphatase B activity peak. To remove this complex, solid $(NH_4)_2SO_4$ was added with stirring to the pooled phosphatase B peak fractions to 100% (saturation). The solution was stirred for 20 min and centrifuged as above. The resulting pellet was resuspended in 20 mL of buffer F, and centrifuged as above to clarify.

Con A Chromatography

The resuspended pellet was absorbed at 0.5 mL/min onto a column (0.5 × 5 cm) of Con A Sepharose which had been preequilibrated with buffer F. The column was connected to the FPLC system, and washed with buffer F until the A_{280} decreased to about 0.05. PEP phosphatase was eluted in a

single peak with a 0 to 500 mM mannopyranoside gradient in buffer F (gradient volume = 30 mL; fraction size = 1 mL). Pooled peak activity fractions were concentrated to about 1.5 mL using an Amicon YM-10 ultrafilter. Binding of the enzyme to Con A suggests that, as has been reported for another acid phosphatase (30), PEP phosphatase is a glycoprotein.

Superose 12 Gel Filtration

Concentrated Con A peak activity fractions were applied at 0.25 mL/min onto a column (1.6 × 50 cm) of Superose 12 (prep grade) which had been connected to the FPLC system and preequilibrated with buffer G (fraction size = 1.0 mL). Pooled peak activity fractions were concentrated to about 0.5 ml as described above, divided into 50 μl aliquots, frozen with liquid N_2, and stored at −80°C. The purified enzyme was stable for at least 6 months when stored frozen.

Table I summarizes the purification of the enzyme. The enzyme was purified about 1700-fold to a final specific activity of about 380 units/mg, and an overall recovery of about 10%.

Gel Electrophoresis and Western Blotting

When the final preparation was denatured and subjected to SDS-PAGE, a single protein staining band migrating with a molecular mass of about 55 kD was observed (Fig. 2).

No immunological cross-reactivity was observed when Western blots of the final preparation (2 μg) were probed with rabbit antibodies directed against either castor bean endosperm cytosolic pyruvate kinase or sorghum leaf PEP carboxylase (data not shown).

Native Molecular Mass Determination

The native molecular mass of the enzyme as estimated by gel filtration of the final preparation on a FPLC prepacked Superose 12 HR 10/30 column was found to be approximately 56 ± 4 kD ($n = 3$).

Heat Stability

The enzyme was relatively heat stable, losing 0%, 20%, and 30% of its original activity when incubated for 4 min at 50, 55, and 60°C, respectively.

Kinetic Properties

Unless otherwise stated all kinetic studies were performed using assay A.

Effect of pH

The pH activity profile of purified PEP phosphatase in the presence of saturating PEP and Mg^{2+} is shown in Figure 3. Under these conditions the enzyme shows a fairly broad pH activity profile with optimal activity occurring at about pH 5.6. All kinetic studies for the purified enzyme were determined at pH 5.6.

Effect of Cations

PEP phosphatase was activated approximately 1.7-fold in the presence of saturating (4 mM) Mg^{2+} (K_a = 1.2 mM). When the reaction mixture contained 10 mM EDTA and no added Mg^{2+} the activity was reduced by about 40%. Ca^{2+}, Co^{2+} and Mn^{2+} were individually tested as Cl^- salts at 4 mM, with no added Mg^{2+}, and found to uniformly activate the enzyme by about 1.4-fold. When tested in place of added Mg^{2+}, 8 mM lead acetate caused 70% inhibition of activity whereas 4 mM $FeCl_3$, $CuSO_4$, or $ZnCl_2$ resulted in complete inhibition of the enzyme. All subsequent kinetic studies of the purified enzyme were conducted in the presence of 4 mM $MgCl_2$.

Substrate Specificity

Enzyme activity was determined using assay B and a wide range of compounds, tested at a concentration of 5 mM unless otherwise specified. The purified enzyme showed no activity with the following compounds: MgAMP, NADPH, *O*-phospho-L-serine, UDP-glucose, fructose 6-P, fructose 1,6-P_2, ribulose 1,5-P_2, dihydroxyacetone-P, glyceraldehyde 3-P, glycerol 3-P, and P-glycolate. No activity was observed with 0.5 mM NaPPi, or with the phosphoprotein, phosvitin (tested at 5 mg/mL).

Table II lists V_{max} and K_m values, along with specificity constants (V_{max}/K_m), and/or phosphatase activity ratios ($v_{0.05\ mM\ [substrate]}/v_{5\ mM\ [substrate]}$) for those compounds which were found to serve as substrates for the purified enzyme. The highest activity was observed with the synthetic substrate pNPP. The K_m for PEP, however, was far lower than the value obtained for any other substrate. Moreover, the enzyme's

Table I. *Purification of PEP phosphatase from B. nigra suspension cells*

Fraction	Volume	Activity	Protein	Specific Activity	Purification	Yield
	ml	*units*	*mg*	*units/mg*	*-fold*	%
Crude extract	1900	1102	4180	0.228		100
Ammonium sulfate	172	1087	2501	0.435	1.9	99
Dialysis	200	1040	720	1.44	6.3	94
S-Sepharose	164	808	164	5.0	17.5	73
Chelating Sepharose	20	383	14	26.5	95	35
Ammonium sulfate	22	229	8.6	27.0	118	21
Con A	42	158	1.6	163	715	14
Superose 12	6.4	115	0.31	380	1670	10

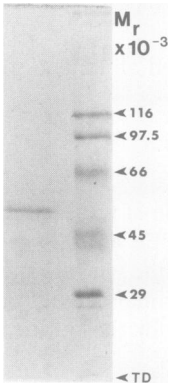

Figure 2. SDS-polyacrylamide mini-gel electrophoresis of purified *B. nigra* PEP phosphatase. The left lane contains 2 μg of the final preparation, whereas the right lane contains 5 μg of the various protein standards described under "Materials and Methods." TD, tracker dye front.

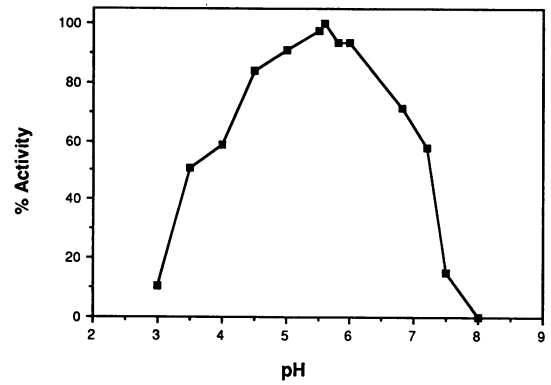

Figure 3. Activity of *B. nigra* PEP phosphatase a function of pH. Sodium acetate-acetic acid, Mes-NaOH, and Hepes-NaOH buffers were used.

specificity constant for PEP was at least six-fold greater than the value determined for any nonartificial substrate. An identical K_m value for PEP was obtained when assay A was used in place of assay B. Hyperbolic substrate saturation kinetics were observed in all cases.

Metabolite and Ion Effects

A wide variety of substances were tested for effects on the purified enzyme using approximately K_m concentrations of PEP (50 μM). The following substances gave no effect (±10% control velocity) on the enzyme activity: NaCl, KCl, DTE, EGTA, NH_4Cl, glutamine, isoleucine, threonine, serine, methionine, valine, and hydroxyproline (all 5 mM); leucine, tyrosine, phenylalanine, tryptophan, arginine, glycine, cysteine, lysine, and proline (all 1 mM); and Ca^{2+}/bovine brain calmodulin (50 μM/1 μM) and trifluoperazine (50 μM). Preincubation of the enzyme with 1 mM *N*-ethylmaleimide for up to 2 min at 25°C also had no effect on enzyme activity. Table III lists those compounds which were found to inhibit PEP phosphatase. The most notable inhibitors were Mo, Pi, F^-, aspartate, glutamate, and various organic phosphates. Also listed in Table III are K_i values for several of the most effective inhibitors. With the exception of F^-, inhibition by these substances was competitive.

Table II. *Substrate Specificity of Purified PEP Phosphatase*

All parameters were determined using assay B as described under "Materials and Methods." Michaelis constants were determined from a Hill plot.

Substrate	V_{max}	K_m	Specificity Constant	Enzyme Activity Ratio
	units/mg	mM	V_{max}/K_m	$\left(\frac{V_{0.05\ mM\ [substrate]}}{V_{5.0\ mM\ [substrate]}}\right)$
PEP	380	0.05	7600	0.48
pNPP	1193	0.57	2093	0.04
Glucose 1-P	304	0.24	1267	0.09
ADP	714	0.74	964	0.03
ATP	452	0.50	904	0.05
Gluconate 6-P	452	0.59	766	0.04
Phytic acid	334	0.82	407	0.03
2,3-P_2-glycerate	110	0.50	220	0.05
3-PGA	255	1.21	211	0
Glucose 6-P	80	2.01	40	0
O-Phospho-L-tyrosine	342	ND[a]	ND	0
O-Phospho-L-threonine	266	ND	ND	0
α-Naphthyl-P	114	ND	ND	0
Fructose 2,6-P_2	53	ND	ND	0
Ribose 5-P	46	ND	ND	0
2-PGA	38	ND	ND	0
ADP-glucose	38	ND	ND	0

[a] ND, not determined.

DISCUSSION

This report describes the purification and characterization of a PEP phosphatase for the first time. *B. nigra* leaf petiole suspension cell PEP phosphatase was purified approximately 1700-fold to a specific activity of 380 μmol/min/mg of protein, and apparent homogeneity. When the synthetic compound pNPP was used as the substrate, the final specific activity was increased to approximately 1,200 μmol/min/mg of protein. This value compares favorably with final specific (pNPP hydrolyzing) activities reported for a range of homogenous plant acid phosphatases (3, 15, 23, 29). Cation exchange and chelating Sepharose chromatography successfully resolved other acid phosphatase activities from the PEP phosphatase activity (Fig. 1). As the phosphatase purified in the present study is not absolutely specific for PEP (Table II), it was important to verify that only one phosphatase was present in the final preparation. The single protein staining band obtained following SDS-PAGE of the final preparation suggests that only one phosphatase was acting on the substrates. Nondenaturing PAGE of the final preparation revealed only a single phosphatase activity staining band (S. Duff, unpublished data). Additional evidence for a single phosphatase was the competitive inhibition of a common substrate in reactions with mixed substrates (Table III).

The final preparation best hydrolyzed PEP among the many substrates tested (Table II). Although the enzyme showed a greater V_{max} with pNPP, MgADP, MgATP or gluconate 6-P, its apparent K_m for these substrates was at least an order of magnitude greater than the value obtained with PEP (Table II). In terms of apparent K_m, glucose 1-P was the next best substrate. However, the affinity of the enzyme for this sub-

Table III. *Effect of Various Substances on the Activity of B. nigra PEP Phosphatase*

Standard assay conditions were used except that the concentration of PEP was subsaturating (0.05 mM). Enzyme activity in the presence of effectors is expressed relative to the control set at 100. K_i values were determined from Dixon plots and patterns of inhibition were evaluated by Lineweaver-Burk plots.

Addition	Concentration Tested	Relative Activity	K_i	Pattern of Inhibition
	mM		μM	
Molybdate	0.1	0	3	Competitive
3-PGA	5.0	0	50	Competitive
Fructose 1,6-P_2	5.0	0	60	Competitive
Pi	0.5	0	85	Competitive
MgADP	0.5	41	180	Competitive
Glyceraldehyde 3-P	5.0	0	450	Competitive
Glutamate	5.0	16	500	Competitive
Aspartate	5.0	39	600	Competitive
NaF	5.0	0	1350	Uncompetitive
Glucose 6-P	5.0	0		
pNPP	5.0	0		
Ribose 5-P	5.0	0		
Fructose 6-P	5.0	0		
PGA	5.0	0		
Glycerol 3-P	5.0	0		
Phytic acid	5.0	0		
Gluconate 6-P	5.0	0		
2,3-P_2-glycerate	5.0	0		
2-PGA	5.0	13		
Glucose 1-P	5.0	13		
Dihydroxyacetone-P	5.0	16		
α-Naphthyl-P	5.0	17		
Ascorbate	5.0	17		
MgATP	0.5	23		
Oxidized glutathione	10.0	33		
Isocitrate	5.0	39		
Mg_2oxalate	5.0	43		
Asparagine	5.0	44		
Mg_2citrate	10.0	50		
Alanine	5.0	51		
Histidine	5.0	51		
EDTA	10.0	58		
Ribulose 1,5-P_2	0.5	72		
Fructose 2,6-P_2	0.5	78		
MgAMP	0.5	89		

strate was still about fivefold lower than that shown for PEP. Thus, although the specificity of this phosphatase is not absolute, we have designated it as a PEP phosphatase because of its: (a) very low apparent K_m for PEP and (b) specificity constant for PEP being at least six-fold higher than the value obtained for any other nonsynthetic substrate (Table II). PEP phosphatase thus joins the ranks of 3-PGA phosphatase (23, 24, 31) and phytase (11) as a class of plant acid phosphatases which exhibit preferential, but nonabsolute, substrate selectivity. Several previously studied plant acid phosphatases have been reported to show a relatively high rate of hydrolysis of PEP (14, 23, 25, 31). However, lack of data on the relative affinity of these enzymes for PEP *versus* other nonartificial substrates makes it difficult to evaluate putative functional similarities between the various phosphatases.

B. nigra PEP phosphatase appears to share several physical and kinetic characteristics with many other plant acid phosphatases (3, 6, 11, 12, 14, 15, 19, 21, 23–25, 29, 31). It displayed a pH optimum of about pH 5.6, was fairly stable at room temperature for short periods, is not a true phosphomonoesterase since it also attacks terminal pyrophosphate bonds, and was inhibited by Pi, F^-, Zn^{2+}, Cu^{2+}, Fe^{3+}, oxidized glutathione, oxalate, ascorbate, and molybdate.

Other aspects of black mustard PEP phosphatase, however, distinguish it from the majority of previously studied acid phosphatases. The enzyme was found to be a monomer with a molecular mass of about 55 kD, which is similar to data reported for a soybean nonspecific acid phosphatase (53 kD monomer) (29) and soybean phytase (50 kD monomer) (11). This contrasts with findings reported for several nonspecific plant acid phosphatases which have a different subunit size and/or are multimeric (6, 15, 21), as well as cane leaf 3-PGA phosphatase (160 kD tetramer) (23) and tobacco leaf P-glycolate phosphatase (81 kD tetramer) (7). Unlike most other acid phosphatases, but similar to tobacco P-glycolate phosphatase (7), and soybean phytase (11), *B. nigra* PEP phosphatase required a divalent cation for optimal activity, was inhibited by EDTA, and was relatively heat-stable. Thus, based upon several physical and kinetic criteria, *B. nigra* PEP phosphatase most closely resembles soybean phytase.

Speculation on the Function of PEP Phosphatase

The apparent K_m value of *B. nigra* PEP phosphatase for PEP was well within probable physiological concentrations of this compound (8) and is equivalent to the value reported for various plant pyruvate kinase preparations (1, 17, and references therein). PEP phosphatase could therefore compete with pyruvate kinase for a common intracellular pool of PEP. Ukaji and Ashihara (28) have demonstrated that the intracellular concentrations of ATP and ADP are more than 16-fold and 8-fold lower, respectively, in Pi-starved *versus* Pi-fed suspension cells of *Catharanthus roseus*. Hence, during periods of prolonged Pi deprivation, PEP phosphatase may endow plants with a capability for the continued conversion of PEP to pyruvate despite the fact that pyruvate kinase activity may become limited by the availability of its substrate ADP. Although energetically 'wasteful,' this biochemical adaptation would provide Pi starved plants with the capacity to utilize Pi provided by ATP and ADP degradation for the most essential metabolic functions, without impairing the conversion of PEP to pyruvate. Consistent with the 'pyruvate kinase bypass' hypothesis is our finding that when Pi-fed *B. nigra* suspension cells were transferred to Pi-free MS media for 7 d, soluble protein concentration decreased about twofold, whereas total PEP phosphatase specific activity increased about 10-fold (S. Duff, unpublished data). This indicates that PEP phosphatase might be Pi starvation inducible. Current investigations are aimed at: (a) using immunological techniques to determine if *B. nigra* PEP phosphatase is Pi starvation inducible, and (b) examining whether other adenylate or Pi dependent reactions of plant glycolysis may also have 'bypass' reactions that are induced during extended periods of Pi starvation.

Possible physiologically meaningful inhibitors of *B. nigra*

PEP phosphatase include Pi, various hexose- and triose-Ps, MgADP, MgATP, and the amino acids glutamate and aspartate (Table III). Competitive inhibition by the various organic phosphates was not unexpected as these compounds are alternative substrates for the enzyme. Potent competitive inhibition by the product Pi (Table III) is in accord with our speculation that PEP phosphatase plays a major role during Pi starvation. The concentration of intracellular Pi in Pi-starved *versus* Pi-fed *C. roseus* suspension cells has been estimated to be approximately 0.03 mM and 6 mM, respectively (28). Thus, PEP phosphatase activity would be promoted when intracellular Pi levels become greatly reduced with Pi starvation. Conversely, any accumulation of Pi caused by Pi resupply would act as a tight regulatory control to prohibit further hydrolysis of PEP by PEP phosphatase, while promoting the synthesis of adenine nucleotides (28) and the consequent reactivation of pyruvate kinase.

Inhibition of PEP phosphatase by glutamate is reminiscent of the reported glutamate inhibition of plant and algal cytosolic pyruvate kinases (1, 17). PEP phosphatase and plant or algal cytosolic pyruvate kinases also share very similar subunit molecular masses, heat stability curves, and apparent K_m values for the substrate PEP (1, 16, 17, 22). Furthermore, in the presence of Mg^{2+} or Mn^{2+}, purified maize leaf PEP carboxylase has been reported to have substantial PEP phosphatase activity (32). Western blots of microgram amounts of purified *B. nigra* PEP phosphatase, however, showed no immunological cross-reactivity with monospecific rabbit polyclonal antibodies prepared against either castor bean endosperm cytosolic pyruvate kinase or sorghum leaf PEP carboxylase. This indicates that PEP phosphatase, cytosolic pyruvate kinase, and PEP carboxylase are probably unrelated proteins.

The subcellular localization of *B. nigra* PEP phosphatase has not yet been established but will be resolved through a combination of immunocytological and subcellular fractionation studies. Although its acid pH optimum is suggestive of a vacuolar localization, significant PEP hydrolysing activity was observed at neutral pH (Fig. 3). Acid phosphatases have been reported to be localized in both the cytoplasm (5, 15) and the chloroplast (7, 19, 24), indicating that an acid pH optimum does not preclude function of a phosphatase in a nonacidic metabolic compartment. Further studies are in progress to examine the distribution, subcellular localization, molecular properties, substrate specificity, and 'coarse' and 'fine' metabolic regulation of plant PEP phosphatases.

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