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SOME PROPERTIES OF PYRUVATE KINASE EXTRACTED FROM *LYCOPERSICON ESCULENTUM*

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; pyruvate kinase; phosphatase; monovalent cations.

Abstract—Pyruvate kinase was extracted from Me_2CO-dried tissue of various parts of tomato plants. Recovery of the enzyme was improved by the inclusion of thiols in the extraction medium, and its stability was increased considerably in the presence of glycerol and to a lesser extent tetramethylammonium chloride. A phosphatase was present in the tissue extracts which hydrolyses phosphoenolpyruvate in the absence of added ADP. ATP inhibited pyruvate kinase but stimulated the phosphatase, while Mg^{2+} stimulated both enzymes. Data obtained suggest that tomato leaf pyruvate kinase has an absolute dependence on monovalent cations for activity, K^+ being the principal activator. The phosphatase was inhibited non-selectively by monovalent cations. The total activity of pyruvate kinase and its concentration on a tissue fresh weight basis was greatest in the leaves, activity increasing with the maturity of the tissue. Less enzyme was present in roots, and least in the fruit.

INTRODUCTION

The properties of pyruvate kinase (pyruvate-ATP phosphotransferase, E.C. 2.7.1.40) from microorganisms and animal tissues have been well documented, but little information is available on the enzyme from plant sources. This has been due mainly to its frequently encountered instability to dialysis and to the presence in plant tissues of a phosphatase which catalyses the ADP-independent hydrolysis of the substrate, phosphoenolpyruvate, so interfering with the assay of pyruvate kinase. For this reason, sources of the enzyme have been largely restricted to seeds, eg., of cotton [1, 2], cucumber [3], pea [1, 2, 4], and to carrot root [4], which generally show a higher ratio of pyruvate kinase to phosphatase activity than other plant tissues. Attempts to circumvent the interference of phosphatase with the assay of pyruvate kinase have included the determination of pyruvate formation from phosphoenolpyruvate in the presence and absence of ADP and subtraction of the contribution from the ADP-independent reaction [5], and the use of molybdate as a specific inhibitor of the phosphatase [3].

Pyruvate kinase from animal tissues was one of the first enzymes shown to require a univalent cation for optimum activity [6, 7]. Miller and Evans [1] subsequently demonstrated that K^+ was necessary for pyruvate kinase activity in eight species of higher plant, NH_4^+ and Rb^+ also being effective. This was confirmed for the enzyme from pea plants [3] and from maize seedlings and maize and cucumber seeds [5].

Apart from an unsuccessful attempt to isolate the enzyme from fruit [8], no studies appear to have been made of pyruvate kinase in the tomato plant. In the present work an improved recovery of the enzyme from tissue was obtained in the presence of thiol compounds, and a number of its properties have been examined, in particular the effects of various cations on its activity. The distribution of the enzyme was determined in various tissues of the tomato plant.

RESULTS AND DISCUSSION

Enzyme extraction

Tomato leaf preparations obtained by extracting the tissue with a medium containing EDTA

Table 1. Effect of some phosphatase inhibitors on the activities of pyruvate kinase and a phosphatase from tomato leaves

Inhibitor	Pyruvate kinase activity*		Phosphatase activity*	
	Pyruvate formed (μmol)	% Inhibition	Pyruvate formed (μmol)	% Inhibition
	0·048	0	0·352	0
1 mM Tris-Mo_2O_7	0·034	28·8	0·345	2·1
10 mM NaF	0·047	2·1	0·271	23·0
10 mM Na_3AsO_4	0·028	41·7	0·124	64·8

* Enzyme activities expressed as μmol of pyruvate formed in 10 min per mg protein-N at 37° in the presence of substrates and salts as described in the Experimental. These prepns. extracted in the absence of thiols.

had low pyruvate kinase activity, accounting for some 12% of the total hydrolysis of the substrate. However, when a thiol was added to the extraction medium, substrate hydrolysis due to pyruvate kinase activity was comparable with that due to phosphatase activity. In a typical experiment, pyruvate kinase activity in the presence of 10 mM EDTA alone was 0·044 μmol pyruvate/mg protein-N/10 min, whereas in the presence of added 10 mM cysteine, 2-mercaptoethanol or dithiothreitol the corresponding activities were 0·28, 0·305 and 0·318 respectively. Because of the enhancement of pyruvate kinase activity extracted in the presence of thiols, in all experiments described here either 2-mercaptoethanol or dithiothreitol was routinely included in the extraction medium. The importance of thiols in the initial extraction of the enzyme was indicated by the finding that the subsequent addition of these compounds to the enzyme assay medium failed to increase the low pyruvate kinase activity of extracts prepared in the absence of a thiol.

Interference by phosphatase

As has been described for a number of other plant tissues [1–5, 9], preparations from tomato leaves contain a phosphatase which interferes with the assay of pyruvate kinase, since it catalyses the ADP-independent hydrolysis of phosphoenolpyruvate (PEP) to pyruvate and orthophosphate. Spencer [10] found that low concentrations of molybdate inhibited the hydrolysis of a series of phosphate esters by tomato leaf phosphatase, although he did not employ PEP as a substrate in these experiments. Evans [3] subsequently used molybdate as a selective inhibitor of phosphatase in a study of pyruvate kinase in the pea plant.

With tomato leaf tissue, however, molybdate was found to inhibit pyruvate kinase to a greater extent than the phosphatase (Table 1). Furthermore, attempts to separate the 2 enzymes by $(NH_4)_2SO_4$ fractionation and by DEAE-cellulose chromatography were unsuccessful. The pH/activity plot of the phosphatase gave maximum activity at pH 4·8–5·0, with no activity above pH 8·0. Since some acid phosphatases are inhibited by fluoride or arsenate [11, 12] these 2 compounds were tested for their effects on the tomato leaf enzymes. The addition of sodium fluoride (10 μmol/ml) produced 23% inhibition of the phosphatase activity with little apparent effect on pyruvate kinase activity (Table 1). Sodium arsenate at the same concentration was a more effective inhibitor of the phosphatase, but also inhibited pyruvate kinase by 42%. Orthophosphate selectively inhibited the phosphatase (Fig. 1), and at 20 mM reduced activity by about 50%. However, in these studies orthophosphate was not routinely included in the assay medium, although if employed as the buffer system it would be expected to reduce the contribution to pyruvate formation caused by the

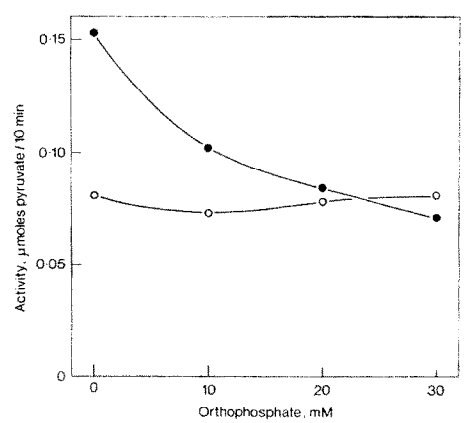

Fig. 1. Effect of orthophosphate on the activities of pyruvate kinase and a phosphatase from tomato leaves. ○ Pyruvate kinase; ● phosphatase.

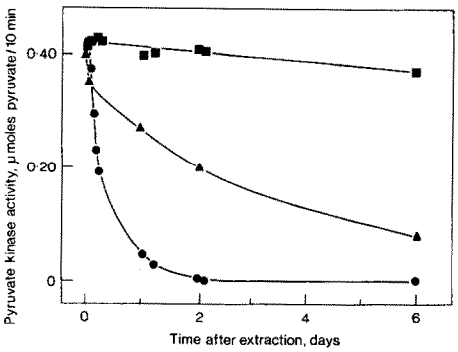

Fig. 2. Stability of pyruvate kinase extracted from tomato leaves. ● No additions to extraction medium; ■ extractions carried out in the presence of 50% (v/v) glycerol, or ▲ 200 mM NMe_4Cl.

phosphatase. In view of the inability to eliminate fully the effect of the latter enzyme, pyruvate kinase activity was determined in terms of the difference in amount of pyruvate formed from PEP in the presence and absence of ADP. Pyruvate kinase activity was highest at pH 7·4–7·6, and a pH of 7·4 was routinely used for assays, since under these conditions the activity of the phosphatase was relatively low.

Enzyme stability

Preparations from many plant tissues rapidly lose pyruvate kinase activity [1, 13], and assays have therefore usually been carried out within 30 min of extraction [13]. Tetramethylammonium chloride was reported to reduce the loss of animal pyruvate kinase activity [14], and recently glycerol has been used to stabilize the enzyme from cotton seed [2].

The enzyme from tomato leaf extracts lost over 50% of its activity after 5 hr at 0° and was almost inactive after 24 hr (Fig. 2.). In the presence of 200 mM NMe_4Cl, 68% of the original activity remained after storage for 24 hr at 0°. Substantial activity was retained in the presence of 50% glycerol, and 89% of the original activity was present after 6 days at 0°. Tissue extracts were either dialysed for 2 hr at 4° in 5 mM Tris–HCl buffer, pH 7·4, containing 10 mM 2-mercaptoethanol, or they were passed through a Sephadex G-25 column. As with pyruvate kinase from a number of other sources [5], the tomato leaf enzyme was unstable to prolonged dialysis.

Effects of adenosine phosphates

In the presence of 1·5 mM PEP the tomato leaf pyruvate kinase was saturated with respect to ADP at a concentration of 1·25 mM, and above this value inhibition occurred. The K_m for ADP, estimated from the saturation curve, was 4×10^{-4} M. This may be compared with Miller and Evans' value [1] of 5×10^{-4} M for pea seed pyruvate kinase in the presence of 1·5 mM PEP [1], and a value of $1{\cdot}55 \times 10^{-4}$ M for the cotton seed enzyme in the presence of 0·05 mM PEP [2]. By contrast, Tomlinson and Turner [4] obtained much lower K_m values for ADP of $1{\cdot}7 \times 10^{-5}$ M (pea seed enzyme) and $5{\cdot}55 \times 10^{-5}$ M (carrot root enzyme) in the presence of 0·033 mM PEP.

ATP, a product of the action of pyruvate kinase, which has been shown to inhibit the enzyme from other higher plants [2, 4], also inhibited tomato leaf pyruvate kinase (Table 2). In the presence of 0·5 mM ATP an inhibition of about 40% was observed. In contrast, 0·5 mM ATP stimulated the tomato leaf phosphatase by 40%. AMP had a

Table 2. Effect of ATP and AMP on the activities of pyruvate kinase and a phosphatase from tomato leaves

Addition to assay medium*	Pyruvate kinase activity†		Phosphatase activity†	
	Pyruvate formed (μmol)	% of control	Pyruvate formed (μmol)	% of control
	0·136[b]	100	0·199[b]	100
0·5 mM ATP	0·083[c]	61·0	0·279[a]	140
2·0 mM ATP	0·050[d]	36·8	0·287[a]	144
0·5 mM AMP	0·133[b]	97·8	0·206[b]	104
2·0 mM AMP	0·148[a]	109	0·196[b]	98·5
Standard error of treatment means	0·003		0·003	

* Tris salts of adenosine 5′-triphosphoric acid (ATP) or adenosine 5′-monophosphoric acid (AMP) used.

† Enzyme activities expressed as in Table 1. Each value is the mean of 3 determinations; those having dissimilar superscripts when compared vertically are significantly different at $P \leq 0{\cdot}05$ by the Studentized range test [15].

small but significant stimulatory effect on pyruvate kinase activity at a concentration of 2·0 mM but not at 0·5 mM. The lower concentration was reported to stimulate the same enzyme from cotton seed, but the effect was reduced in the presence of chloride ions [2]. With the tomato leaf enzyme Tris/HCl buffer was used in the assay, and this might therefore account for the lack of response to low concentrations of AMP. Activation by AMP, coupled with inhibition by ATP, would be expected to cause pyruvate kinase to respond to energy charge [16, 17], as in the case of the enzyme from cotton seed, where it has been proposed as having a regulatory role [2].

Effects of cations

Addition of magnesium sulphate to enzyme extracts stimulated both pyruvate kinase and the phosphatase, although in neither case was an absolute dependence on Mg^{2+} demonstrated (Fig. 3). Maximum activities of the 2 enzymes were obtained in the presence of 10 mM and 30 mM $MgSO_4$, respectively.

The relation between pyruvate kinase activity and potassium concentration was determined in several experiments. Most of the potassium present in the leaf tissue was removed during the preparation of the Me_2CO powder. When assayed in the absence of added salts, pyruvate kinase activity in extracts from Me_2CO powders was about 10% of that obtainable in the presence of an optimum

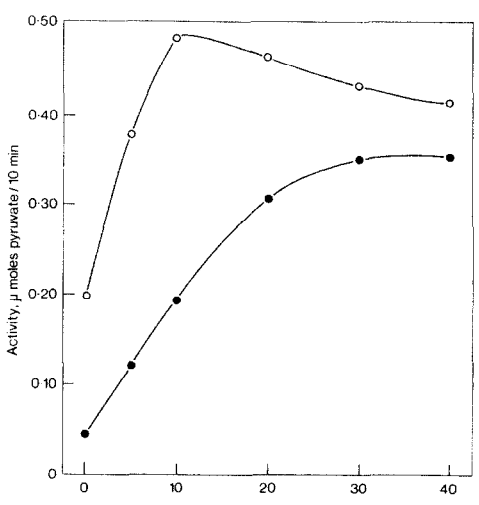

Fig. 3. Activation of tomato leaf pyruvate kinase and a phosphatase by Mg^{2+}. ○ Pyruvate kinase; ● phosphatase.

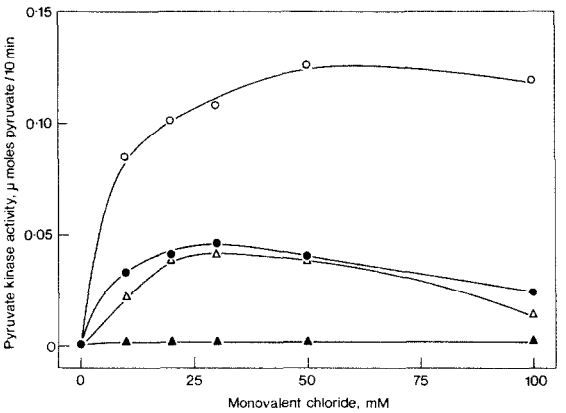

Fig. 4. Activation of tomato leaf pyruvate kinase by monovalent cations. Additions to assay medium; ○ KCl; ● NaCl; △ NH_4Cl; ▲ LiCl. Values on each curve were obtained from an individual enzyme preparation and were corrected for differences in enzyme concentration (determined in the presence of 50 mM KCl).

concn of KCl. Prolonged dialysis of extracts led to complete loss of activity. Moreover, after a brief dialysis of 2–4 hr against 10 mM Tris–HCl buffer, pH 7·4, containing 10 mM 2-mercaptoethanol, the potassium content was reduced to give a concentration in the complete assay system of about 0·3 mM K^+, and the resultant pyruvate kinase activity was less than 5% of the maximum attainable. This strongly suggests that the enzyme has an absolute dependence on K^+ for activity.

Maximum pyruvate kinase activity was obtained in the presence of 50 mM KCl (Fig. 4). At much higher concentrations a decrease in reaction rate occurred, and at 300 mM KCl a reduction of 28% was observed. The concentration of KCl required to produce half the maximum reaction velocity (K_A) estimated from the saturation curve was 7×10^{-3} M. As shown in Fig. 4, addition of NH_4Cl or NaCl to the assay system in place of KCl also resulted in considerable stimulation of pyruvate kinase activity. The maximum effects of these salts occurred at a concentration of 30 mM, but the stimulatory effects were only about one-third of that produced by KCl. LiCl was found to be ineffective as an activator of the enzyme. Responses to monovalent cations similar to those described above have been reported by Miller and Evans for the enzyme from pea seed [1]. However, these authors obtained a much smaller K_A for KCl of $2{\cdot}4 \times 10^{-3}$ M, whereas the work of Kachmar

and Boyer [18] with the rabbit muscle enzyme indicated a more comparable value of 11×10^{-3} M.

In contrast, the hydrolysis of PEP by the phosphatase was inhibited non-selectively by the monovalent salts tested, about 60% of the original activity being obtained in the presence of concentrations of 100 mM in the assay system (Fig. 5). *In vitro*, therefore, monovalent cations, especially K^+, affected the efficiency of energy conservation during the hydrolysis of PEP, since they increased the rate of production of ATP via the pyruvate kinase reaction and simultaneously reduced the rate of ADP-independent hydrolysis caused by the phosphatase.

Distribution of pyruvate kinase in tomato plant tissues

Table 3 shows the pyruvate kinase activity of various parts of tomato plants (cv. Amberley Cross). Enzyme activity, expressed in terms of unit wt of fresh tissue or of protein-N, or on a per plant part basis, increased with leaf maturity. Activities expressed on a fresh tissue basis were comparable in roots and shoot apices, and were lowest in fruit. When expressed in terms of protein-N, the activity in fruit was similar to that in fully expanded leaves. Although the highest activity, on a protein-N basis, was found in the roots most of the enzyme was located in the fully expanded leaves. Since pyruvate kinase has a requirement for K^+, it would be expected that the potassium status of the plant would be an important factor in influencing the activity of the enzyme in tissues. Studies are in progress to determine in more detail the interactions of monovalent cations in relation to pyruvate kinase activity, and to examine the influence of potassium nutrition and the potassium status of the plant on the foliar distribution of the enzyme.

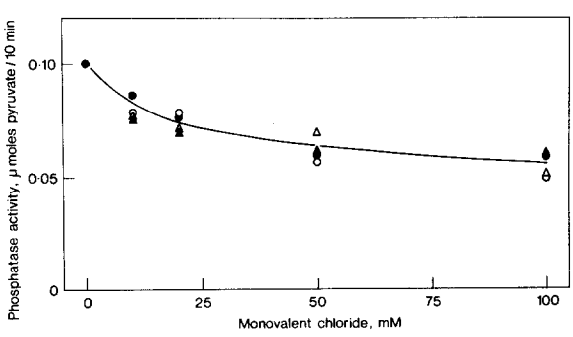

Fig. 5. Effect of monovalent cations on tomato leaf phosphatase. Additions to assay medium; ○ KCl; ● NaCl; △ NH_4Cl; ▲ LiCl. Values obtained in the presence of each monovalent chloride were obtained from individual enzyme preparations and were corrected for differences in initial enzyme activity (in the absence of added salts).

Table 3. Distribution of pyruvate kinase activity in tomato plant tissues

Plant part	Pyruvate kinase activity (μmol pyruvate formed)* per *g* fresh tissue	per mg protein-N	per fraction†
Shoot apex	2·54[c]	1·13[d]	2·54 ± 0·14
Expanding leaves	4·75[b]	3·40[c]	170·1 ± 0·83
Fully-expanded leaves	6·18[a]	5·27[b]	414·0 ± 57·1
Red fruit	0·59[d]	5·70[b]	27·9 ± 3·6
Roots	2·05[c]	9·22[a]	96·6 ± 0·8
Standard error of treatment means	0·40	0·31	

* Each value is the mean of at least three determinations; those having dissimilar superscripts when compared vertically are significantly different at $P \leqslant 0{\cdot}05$ by the Studentized range test [15]. † Figures in this column derived from the product of the values per g fresh tissue and the total fr. wt of each fraction (arbitarily choosing 1 g fr. wt of shoot apical tissue and taking the average fr. wt of the pericarp tissue of a single red fruit), together with the individual standard error associated with each mean.

EXPERIMENTAL

Preparation of plant material. Tomato plants, cv. Amberley Cross, were grown in sand in a glasshouse with mean day and night temps of 24 and 17·8°, respectively. The plants were supplied with a nutrient soln containing 2·0 me K^+/l, as described elsewhere [19]. After 8 wk the plants were dissected into 4 fractions, viz. shoot apices (≃ 2 g fr. wt), expanding leaves (5–9th inclusive from the shoot apex, defining the first as that being 1 cm or longer), fully expanded leaves (10–14th inclusive) and roots. Some plants from the same trial were terminated above the 1st truss and grown until the fruit were ripe. Representative samples of red fruit were selected for analysis and the pericarp tissue was used in the extraction procedure. Fresh, chilled tissue was homogenized with 10–20 vol. Me_2CO cooled to −15°, the temp. throughout being kept below 5°. The homogenate was filtered and the filter cake washed with Me_2CO at −15° until the filtrate was colourless and then dried *in vacuo*. Samples of the powder were used immediately for enzyme extraction, or after a brief period of storage at −15°.

Enzyme extraction. Fifteen ml of 0·1 M Tris–HCl buffer, pH 7·8, containing 10 mM EDTA and 10 mM dithiothreitol were added to 0·5 g of Me_2CO-dried powder and vigorously agitated for 20 min at 4°. The suspension was squeezed through 4 layers of muslin and the filtrate centrifuged at 20000 g for 10 min at

2°. For studies of enzyme properties, the preparations were either dialysed for 2 hr at 4° against 5 mM Tris–HCl buffer, pH 7·4, containing 10 mM 2-mercaptoethanol, or they were passed through a 1·8 cm × 22 cm column of Sephadex G-25. For studies of enzyme distribution in the plant, undialysed preparations were used without further treatment.

Enzyme assays. Pyruvate kinase act. of tissue preparations was assayed by determining the ADP-dependent formation of pyruvate from phosphoenolpyruvate (PEP), and phosphatase act. was obtained by determination of the pyruvate formed in the absence of ADP, the procedure being an adaptation of that of Kachmar and Boyer [18]. Assay solns contained the following constituents (in μmol): Tris–HCl buffer, pH 7·4, 50; PEP (monocyclohexylamine salt), 1·5; $MgSO_4$, 10; KCl, 50; ADP (Tris salt), 1·25; together with enzyme preparation in a final vol of 1 ml. PEP was omitted from control solns. The mixture was incubated for 10 min at 37° and the reaction stopped by the addition of 1 ml of 0·025% 2,4-dinitrophenylhydrazine in 2N HCl. Pyruvate was determined by the method of Kachmar and Boyer [18]. The pyruvate formed by phosphatase in the absence of ADP was subtracted from that formed in the presence of ADP to obtain the amount produced by pyruvate kinase.

Gel filtration. Sephadex G-25 was suspended in 0·1 M Tris–HCl buffer, pH 7·4, and allowed to sediment in a column of 1·8 cm i.d. to a height of 22 cm. The column was used in a cold room at 4°.

Protein-N determinations. The protein-N content of enzyme extracts was determined using a Technicon AutoAnalyzer [20] after precipitation with 10% trichloroacetic acid and digestion with H_2SO_4, using Se as catalyst [21]. The contribution of nucleic acid to the nitrogen values was estimated by the method of Warburg and Christian [22], and corrections were made where necessary.

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