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PURIFICATION AND PROPERTIES OF PHOSPHOFRUCTOKINASE FROM FRUITS OF *LYCOPERSICON ESCULENTUM*

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato fruit; phosphofructokinase; affinity chromatography; molecular form.

Abstract—A procedure is described using affinity chromatography on Blue Sepharose and on an immobilized ATP column by which phosphofructokinase has been purified by 260-fold from tomato fruits. The properties of the enzyme are affected by the pH at which the preparation is made and maintained. At the pH optimum, pH 8.0, the enzyme is very heterogeneous with up to three forms present differing in MW. At pH 7.5 a single major form of MW 180 000 is present, and evidence that raising the pH to 8.0 promotes dissociation of the enzyme is discussed.

INTRODUCTION

It is generally agreed that the control of glycolysis in plants is dominated by the interaction between phosphofructokinase (PFK) and pyruvate kinase although other control points may be involved to some extent [1]. PFK has been implicated in the control of the increased glycolytic flux [2, 3] which accompanies the rise in respiration during the ripening of the climateric fruits [4]. Although PFK from animal and microbial sources has been extensively purified and its properties studied in some detail [5, 6] reports of similar studies on the enzyme from plant sources are few. This is partially due to the fact that the plant enzyme is often rather unstable and hence difficult to purify [7]. The present paper describes the use of affinity chromatography to purify the enzyme. This work forms part of a study of the kinetic regulation of the enzyme during the ripening and senescence of tomato fruits [8].

RESULTS AND DISCUSSION

Purification of PFK from tomato fruits

Table 1 shows the application of various procedures for the partial purification of PFK from tomato fruits. Initially various methods of concentrating the crude extract prior to chromatography were tried but as shown in Fig. 1, PFK is inhibited by many inorganic ions such as SO_4^{2-}, NO_2^-, NO_3^- and Cl^-, but the inhibition by Cl^- was much less than with the

Table 1. Purification of PFK (all purifications done at pH 7.5)

Preparation	Wt of tissue (g)	Vol. (ml)	Act./ml (pkat)	Protein (μg/ml)	Sp. act. (pkat/μg protein)	Total act. (pkat)	Yield (%)	Purification
1.								
Crude extract	150	365	32.3	823.4	0.039	11800	100	1
Blue Sepharose		18	492.3	91.4	5.4	8860	75.2	137
ATP-Sepharose		9	630.8	61.3	10.3	5680	48.2	262
2.								
Crude extract	100	224	30.8	760	0.04	6900	100	1
Blue Sepharose		18	302	77	3.9	5440	78.8	96.8
3.								
Crude extract	200	500	36.2	868	0.042	18100	100	1
DEAE – MgATP		54	57.2	255	0.224	3090	17	5.4
4.								
Crude extract	180	392	40.8	642	0.0635	16000	100	1
DEAE + MgATP		48	186	310	0.6	8930	55.8	9.5

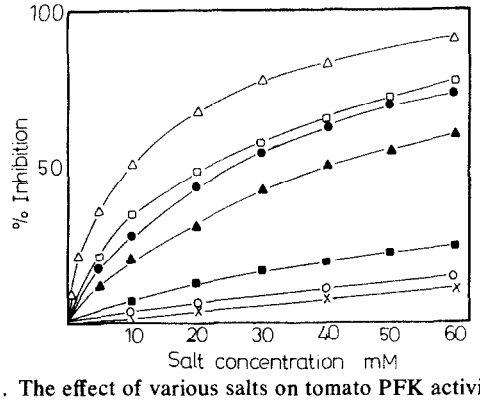

Fig. 1. The effect of various salts on tomato PFK activity at pH 8.0. × NaCl, ■ KCl, ○ NH_4Cl, ● Na_2SO_4 or K_2SO_4, □ $NaNO_2$, ▲ $(NH_4)_2SO_4$ and △ $NaNO_3$.

other ions. The inhibition by ammonium sulphate ruled out the conventional ammonium sulphate method for concentrating the enzyme since the inhibition by ammonium sulphate was only partially reversed by desalting on a column of Sephadex G-25. Recoveries of activity following ammonium sulphate precipitation and desalting were of the order of 10% and it appeared that the enzyme becomes very unstable during the procedure. Other methods for concentrating the enzyme such as isoelectric precipitation and concentration by ultrafiltration were tried but discarded since they led to rather variable recoveries of PFK activity. Precipitation with polyethylene glycol (PEG 4000) was effective even though high concentrations (up to 65%) were needed for a good recovery of activity. PEG precipitation was used where it was essential to apply the enzyme fraction in a small volume prior to chromatography, i.e. during gel permeation chromatography on Ultrogel AcA 34, but in general it was found that the crude enzyme fraction could be applied directly to other chromatographic reagents such as DEAE-cellulose or the affinity adsorbents if it was first desalted. This was achieved by rapid dialysis using a hollow fibre ultra-filtration system.

Chromatography on DEAE-cellulose using a linear gradient of salt for elution led to 5–10-fold purification of PFK but this was accompanied by an 80% loss of activity which could be partially prevented by including ATP and Mg^{2+} in the elution buffers (Table 1; sections 3 and 4). In view of this instability alternative methods for the purification of PFK were sought. Blue Sepharose CL6B was found to retain PFK from crude extracts of tomato which had been dialysed against 5 mM Tris buffer. The PFK fraction could be recovered from the column by specific desorption with the substrate of the enzyme, MgATP (10 mM ATP + 30 mM $MgCl_2$ in 5 mM Tris buffer) leading to a 97–137-fold purification with the recovery of 75–79% of the applied activity.

The PFK peak from Blue Sepharose CL6B was dialysed against dilute buffer and then further purified on a column of immobilized ATP, 8-(6-amino hexylamino)-ATP coupled to Sepharose which was found to retain tomato PFK at low salt concentration. The PFK activity could be recovered by elution with ATP (10 mM) and 30 mM $MgCl_2$ in 5 mM Tris buffer pH 7.5. The combined use of Blue Sepharose and ATP-Sepharose led to a 260-fold purification of PFK with an overall recovery of 49% (Table 1; section 1). The most highly purified fraction of PFK had a sp. act. of 10 pkat/μg protein which compares favourably with the values found for the enzyme purified to homogeneity from rabbit skeletal muscle [9].

PAGE of the PFK fraction after chromatography on hexyl ATP–Sepharose showed one major protein band with a second protein component present only in trace amounts (Fig. 2). Using a PFK specific assay [19], it was shown that PFK activity is associated with the major protein band. These results indicate that the PFK preparation has been purified close to homogeneity.

Properties of purified enzyme

The pH optimum of the enzyme was 8.0–8.25 with a relatively steep fall in activity on the acid side of the optimum so that at pH 7.5 the activity was half that at the maximum and at pH 7.0 it was only about 15% of the maximum value. Increasing the concentration of ATP from 1 to 2 mM and of fructose-6-phosphate from 2 to 10 mM had no effect on the pH optimum. The pH of extraction medium and the pH at which tomato extracts are stored appears to have an important effect on both the catalytic activity and molecular properties of PFK. Extracts prepared with 0.2 M Tris–HCl at pH 8.0 were found to be much less stable during storage at 0–4° than extracts prepared at pH 7.5. The enzyme is less stable in phosphate buffer compared with Tris buffer at either pH 7.5 or 8.0. The

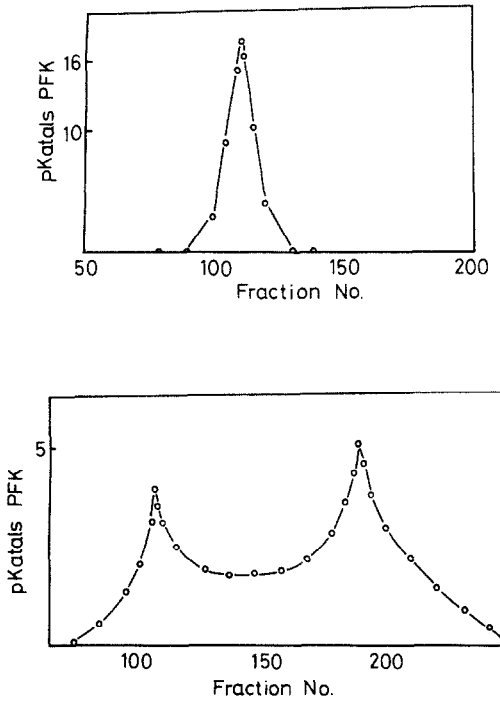

Fig. 2. Separation of PFK activity on Ultrogel AcA 34. Upper curve—extract prepared and maintained at pH 7.5. Lower curve—extract prepared and maintained at pH 8.0.

inclusion of MgATP in the buffer especially at pH 7.5 tends to reduce the loss of activity as does the inclusion of DTE. The effect of DTE on the stability of the enzyme is an important one but even the use of DTE does not alter the relative instability of the enzyme at pH 8.0 compared with 7.5. Tomato PFK is sensitive to freezing and even at pH 7.5 virtually all the activity is lost during a period of storage at −20° for 24 hr. Tomato PFK is relatively heat labile. Three min at 60° is sufficient to cause complete inactivation of the fraction after chromatography on ATP–Sepharose. However, the same heat-treatment had only a small effect on the enzyme prepared by chromatography on DEAE-cellulose in the absence of MgATP. In this case even treatment at 100° for 5 min only reduced the activity by 70%. Differences in the degree of thermal lability of PFK prepared under different conditions were noted [8] and these differences which may be related to changes in the molecular form of the enzyme will be discussed later in this paper.

PFK extracted from tomato fruits at pH 8.0 and applied to a column of Ultrogel AcA 34 after PEG precipitation was shown to be very heterogeneous in MW (see Fig. 2). Activity was spread widely across the chromatogram with two major peaks of activity; one eluting in a volume of 111 ml and the other in 190 ml. It was found that if MgATP was included in the elution buffer there was an improvement in the recovery of total activity from the column from 28% in its absence to 58% in its presence. However, the degree of heterogeneity was not reduced and in fact an additional peak eluting early (elution volume 90 ml) in the separation was observed. The peaks eluting at 90, 111 and 190 ml were designated UG 3 UG 2 and UG 1, respectively. If the Ultrogel chromatography was carried out with extracts prepared and maintained at pH 7.5 much less heterogeneity was observed. At pH 7.5 a single symmetrical peak of activity was observed corresponding in elution position to peak UG 2 (Fig. 2). The inclusion of MgATP in the elution buffer at pH 7.5 increased the recovery from the column from 55 to 95% and led to the appearance of a small peak eluting in the position corresponding to UG 3. This small fraction, UG 3 appeared only in the presence of MgATP and was found to be very labile which has made the study of its properties difficult.

The Ultrogel column was calibrated by determining the elution volume of proteins of known MW [8] and estimates of the MWs of the three fractions of PFK activity gave values of 360000, 180000 and 35000 respectively for UG 3, UG 2 and UG 1. UG 1 was further studied by chromatography on Ultrogel AcA 54 with a fractionation range more appropriate to a small protein such as UG 1 and the estimate of 35000 was confirmed.

The analysis of the highly purified PFK fraction from ATP-Sepharose on Ultrogel AcA 34 at pH 7.5 led to the elution of a single peak of activity corresponding to UG 2 while the same fraction chromatographed at pH 8 gave two peaks of activity in the positions of UG 1 and UG 2. Altering the pH from 7.5 to 8.0 appeared to promote the appearance of the low-MW fraction UG 1. If preparations were stored at pH 8 for several days at 0–4° there was a progressive increase in UG 1 and the disappearance of UG 2 until after 4 days all of the oligomeric form had disappeared.

The highest MW form (UG 3) is present only in very small quantities and is very labile. The ratio of MWs suggests that UG 3 could be a dimer of the form UG 2 which is generally the major form present. There is no simple stoichiometric relationship between UG 2 and the smallest form UG 1. UG 1 is the lowest MW form found in the tomato and may be a monomeric form of the enzyme while UG 2 and 3 may be oligomeric forms composed of UG 1 units.

Most studies on plant PFK have used relatively crude preparations. This is necessitated in some cases by the extreme lability of some plant PFK preparations [7] but in other cases it was found desirable to use the crude preparation because of the observation that the regulatory properties of the enzyme may change on storage even though the catalytic activity was retained [10]. There are obvious problems in attempting the purification of an enzyme as complex as PFK in its molecular form and in its kinetic behaviour [5, 6]. We have purified tomato PFK by a factor of 260-fold by combined affinity chromatography on Blue Sepharose and on an immobilized form of ATP. Affinity probes have been used in the purification of PFK from animal sources [11, 12] but this is to our knowledge the first successful application of this material to a plant PFK [8].

The tomato enzyme differs from other plant PFKs in a number of its properties. It is for instance relatively stable while a preparation of the PFK from potatoes was found to lose up to 90% of its activity within 10 hr of preparation [7]. Tomato PFK is heat labile while other PFKs are heat stable, a property which has been used in the purification of the enzyme from certain plant sources [2, 13]. The pH optimum of the tomato enzyme at 8.0–8.25 is similar to that of some plant and animal PFKs but differs from the pea seed enzyme [14] which has a peak at pH 7.0 in addition to one at pH 8.0 with a minimum of catalytic activity at about pH 7.5.

Heterogeneity in molecular form is a common feature of PFK isolated from animal sources [5, 6, 9] and evidence for different isoenzymes of PFK in the tomato [15] has been presented. In the present paper, tomato PFK has been shown to exist in a single stable oligomeric form at pH 7.5 but raising the pH to 8.0 or above promotes dissociation and heterogeneity.

EXPERIMENTAL

Materials and methods. Tomatoes (*Lycopersicon esculentum* var. Eurocross BB) were obtained from plants grown in the Food Research Institute greenhouses.

All biochemicals were purchased from Boehringer, and were of the best quality available. Tris, Hepes, DTE and Sephadex G-25 were purchased from Sigma. Polyethylene glycol, Polyclar AT and all other chemicals were of analytical grade and were purchased from BDH. DE-52 was obtained from Whatman, and Ultrogel AcA 34 was a product of LKB. Blue dextran, Blue Sepharose (CL-6B) and cyanogen bromide activated Sepharose were obtained from Pharmacia.

For assaying phosphofructokinase activity, the reaction mixture contained the following in a 1-ml cuvette: 16 μmol NADH, 5 μmol $MgCl_2$, 3 μmol ATP, 2 μmol FBP, 0.3 units GDH, 2 units TIM and 0.4 units of aldolase.

The reaction which was carried out at 25° was initiated by the addition of fructose-6-phosphate. The rate of the reaction was followed by measuring the decrease in fluorescence as NADH was reoxidized using an Eppendorf fluorimeter with a primary filter of 313–366 nm and a secondary filter Hg 420–300 nm. A specially built attenuator was used to provide a suitable signal to a potentiometric recorder (Bryans type 23 000) with sufficient back-off so that the recorder could be used at high sensitivity. This system showed linearity between NADH concentration and the recorder output.

Extraction of PFK from tomatoes. The extraction medium consisted of 0.2 M Tris–HCl buffer containing 2 mM EDTA and 5 mM DTE at the appropriate pH (either 7.5 or 8, see text). The tomatoes at the breaker stage were cut and the outer wall of the pericarp together with the epidermis were taken. Two ml of the extraction medium were added per g of tissue together with polyclar AT (50 mg/g tissue). The tissue was homogenized in an Ultraturrax homogenizer (type TP18/2 from Janke and Kunkel) at 140 V for about 1 min. The homogenate was filtered through Miracloth and the resulting filtrate centrifuged at 35000 *g* for 25 min in a pre-cooled centrifuge. The supernatant represents the crude extract. Dialysis of the crude extract was carried out either using dialysis bags or by ultrafiltration using a hollow fibre system (type b HFU/1 from Biorad). The dialysis buffer was 5 mM Tris–HCl at either pH 7.5 or 8 containing 2 mM EDTA and 1 mM DTE. All these operations and the later purification of the enzyme were carried out at 0–4° using buffers containing 1 mM DTE. For chromatography on DEAE-cellulose the dialysed crude extract was applied directly to a column (12 × 1.5 cm) at a rate of 55 ml/hr. The column was washed with 50 ml of 5 mM Tris–HCl containing 2 mM EDTA and 1 mM DTE (buffer A). The enzyme was eluted using a linear gradient of KCl (0–0.4 M) in the same buffer. Whenever Mg and ATP were used, they were added to the buffer A to a concn of 1 mM ATP and 5 mM Mg^{2+} and the pH was re-adjusted to 7.5.

For Blue Sepharose chromatography, the dialysed crude extract was applied to the column of Blue Sepharose CL6B (10 × 1.5 cm) at the rate of 30 ml/hr. After washing the column with 50 ml of buffer A, the enzyme was eluted using 50 ml of 10 mM ATP and 30 mM $MgCl_2$ dissolved in the same buffer at pH 7.5. Fractions of 1 ml were collected and the rate of flow was reduced to 15 ml/hr to improve the separation. Other workers have applied this method to the potato enzyme with satisfactory results [16].

For ATP-Sepharose chromatography, 8-(6-amino hexyl amino)ATP was prepared according to the method of ref. [17]. Then it was coupled to Sepharose using cyanogen bromide activated Sepharose. The resulting gel was packed in a column (10 × 0.9 cm) and then equilibrated with 5 mM Tris–HCl at pH 7.5 containing 2 mM EDTA and 1 mM DTE.

This method was used as the final stage for purifying the enzyme. The peak fractions from the Blue Sepharose column were dialysed against a large volume of buffer A to remove $MgCl_2$ and ATP. The resulting dialysate was applied to the ATP–Sepharose column at the rate of 30 ml/hr. After washing the column with 50 ml of the buffer, the enzyme was eluted with 10 mM ATP and 30 mM $MgCl_2$ in buffer A.

The PFK preparations were checked for the presence of some interfering enzymes including phosphoglucoisomerase, fructose-1,6-diphosphatase, phosphoglucomutase and ATPase and which were found to be absent from the most purified fractions.

The purity of PFK preparation after hexyl-ATP-Sepharose was studied by discontinuous PAG electrophoresis using 5 and 7% gel rods at pH 7.5. The protein components were stained with Coomassie Blue [18] and the localization of PFK was determined by an assay linked to the deposition of insoluble formzan [19].

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