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PHOSPHOFRUCTOKINASE AND RIPENING IN *LYCOPERSICON ESCULENTUM* FRUITS

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato fruit; phosphofructokinase; climacteric; senescence; fruit ripening.

Abstract—The relative activity and the kinetic properties of phosphofructokinase (PFK) during the various stages of tomato ripening were investigated. There were no significant changes in the extractable activity of the enzyme during ripening but there was an apparent change in the molecular form of the enzyme as the fruits entered the climacteric and senescence stages. While the enzyme extracted from preclimacteric fruit existed solely in an oligomeric form, that extracted from fruits in the later stages of ripening was present as a mixture of the monomeric and oligomeric forms. Changes in the regulatory properties of the enzyme extracted at the various stages of ripening were explicable in terms of the dissociation of the oligomeric form of the enzyme to smaller units. PFK from the preclimacteric fruit is more resistant to inhibition by citrate and salts than the enzyme from the post climacteric fruit. On the other hand, the preclimacteric enzyme is stimulated by Pi and ADP while the post climacteric enzyme is not. The significance of these effects in relation to the physiology of tomato ripening and senescence is discussed.

INTRODUCTION

In certain fruit, ripening is preceded by a sudden upsurge in respiration termed the respiration climacteric. This increase in respiration is associated with the autocatalytic production of ethylene, marks the onset of fruit senescence and generally precedes the visible changes in the colour, flavour and texture associated with ripening [1]. Glycolysis has been found to be enhanced during the climacteric rise in a number of fruits such as bananas [2], avocados [3] and tomatoes [4]. The decrease in fructose 6-phosphate concentration and increase in fructose 1,6-bisphosphate during the climacteric has been taken as indicating an increase in the activity of phosphofructokinase (ATP:D-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11, hereafter PFK) and the increase in glycolytic activity during the climacteric [2–4]. However, there is very little understanding of the way the activity of this enzyme is regulated [5, 6] and its role in the ripening of fruits is uncertain [2, 7–9].

In our previous work, PFK from the tomato has been purified and its kinetic and regulatory properties were extensively studied [10–13]. The aim of the present work is to examine the properties of PFK in relation to the changing physiological state of the tomato fruit during ripening.

RESULTS

The relative activity of PFK and the protein content of extracts prepared from fruit at the different stages of ripening were determined and the results are shown in Table 1. There is a progressive decline in both the protein content and relative activity of PFK whether expressed per gram of fresh weight or per microgram of protein as ripening proceeded.

Since it has been shown earlier that PFK from tomatoes, at the 'breaker' stage, exists in an oligomeric form (M_r 180 000) [10], the nature of the enzyme during the various other stages of ripening was examined using gel permeation chromatography. Figure 1 (a–d) shows the elution pattern of PFK from the various stages of ripening on the same Ultrogel AcA 34 column and under identical

Table 1. Activity of PFK at various stages of tomato ripening (± standard deviations of the mean)

Stage	No. of investigations	Protein (μg/g fr. wt)	Sp. activity (pkatals/ μg protein)	Ratio monomer/ total PFK
Green	4	2260 ± 24	0.085 ± .004	0
Breaker	4	1920 ± 17	0.076 ± .003	0
Orange	3	1670 ± 12	0.073 ± .002	0.36
Red	3	1600 ± 13	0.073 ± .004	0.52

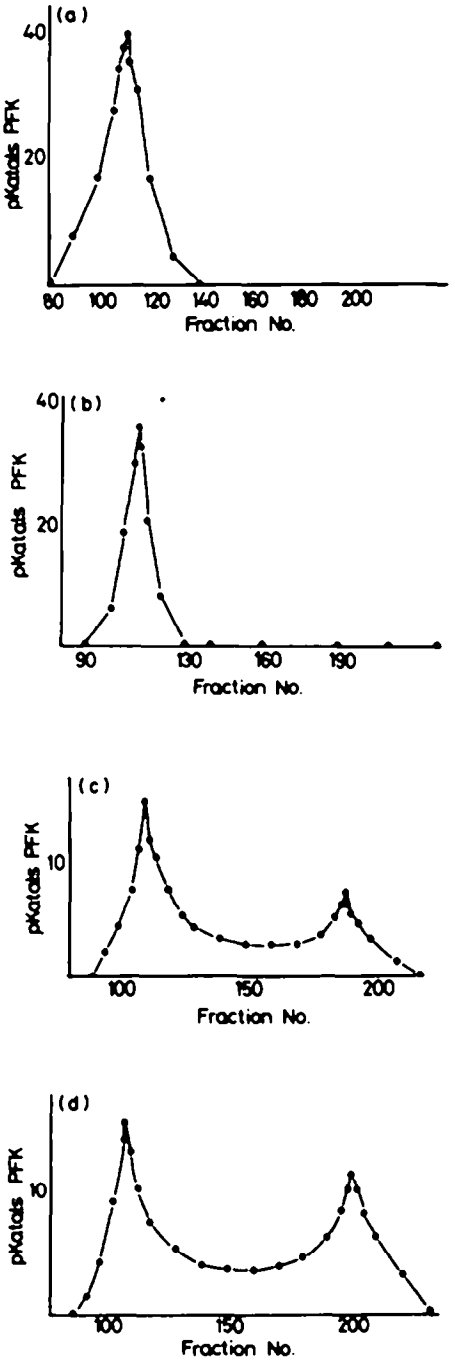

Fig. 1. Elution of PFK on Ultrogel AcA 34 for the enzyme preparation from (a) green, (b) breaker, (c) orange and (d) red tomatoes.

conditions. The enzyme eluted as one single peak corresponding to the oligomeric form of PFK for both the green and breaker stage. On the other hand, for the orange and red stage there were two peaks of PFK activity corresponding to the oligomeric and monomeric forms of PFK.

Table 1 shows the ratio of the monomeric to total PFK in relation to the various stages of ripening. It is clear that this ratio increases during the period following the climacteric peak in respiration and it continues to increase as the fruit senescences.

Changes in regulatory properties with ripening

Studies on the kinetic and regulatory properties of PFK were carried out using dialysed crude extracts prepared from the fruit at the various stages of ripening. These studies will be presented in comparison with those conducted on both the oligomeric and monomeric forms of PFK separated on Ultrogel columns. To obtain a mixed population of the monomeric and oligomeric forms of PFK, the purified oligomeric form was subjected to incubation at an elevated pH to induce its dissociation and assayed immediately [10]. Table 2 summarizes the effect of different modulators on the activity of PFK extracted from the various stages of tomato ripening compared with their effect on the molecular forms of the enzyme.

The results show that the stimulation of PFK activity by Pi decreases as the tomatoes ripen. Pi had a slight inhibitory effect on the monomeric form of the enzyme while it exhibited its maximum stimulation to the oligomeric form. Consequently, the difference in Pi effect on PFK from the various stages of plant growth can be explained in terms of a change in the molecular form of the enzyme. A similar result is observed for the effect of ADP on the enzyme from the various stages of plant growth. Table 2 shows also that the monomeric form of tomato PFK is more sensitive to inhibition by citrate and ammonium sulphate than the oligomeric form of the enzyme. The inhibition by either citrate or ammonium sulphate increases as the tomatoes pass from the green to red stage and this is consistent with the view that the oligomeric form of the enzyme dissociates as ripening proceeds.

Effect of PEP

The effect of PEP on PFK activity was difficult to study in crude extracts since addition of PEP to the assay mixture gave rise to side reactions due to the presence of interfering enzymes. However, the effect of PEP on PFK activity was investigated on the purified oligomeric and monomeric forms of PFK individually [12]. PEP is a very potent inhibitor of both forms of the enzyme with the concentration required for half maximal inhibition equivalent to 9–10 μM but the monomeric form of PFK was inhibited more by PEP than the oligomeric form [12]. It could be concluded here that PFK from the green and breaker stage is more resistant to PEP inhibition than the enzyme from the orange and red stages.

The kinetics of PFK with respect to its substrates

The kinetic studies of PFK from the various stages of plant growth with respect to Mg^{2+} and ATP showed that the enzyme is inhibited by the free trinucleotide and Mg^{2+} was able to alleviate this inhibition [11]. Analysis of the kinetic data revealed that these kinetics follow the Michaelis–Menten pattern. In extracts prepared at the various stages of ripening, there was very little change in V_{max} and K_m remained constant at 0.46 mM for ATP and 0.84 mM for Mg^{2+} [11].

The kinetics of PFK were investigated for its other substrate F6P for extracts of tomatoes at various stages of

Table 2. Effect of different modulators on tomato PFK activity

Enzyme preparation	% Change in activity by			
	10 mM Pi	0.5 mM ADP	2 mM citrate	25 mM ammonium sulphate
Green stage crude extract	+59	+42	−22.4	−9.8
Breaker stage crude extract	+51.2	+39.4	−23.2	−12.9
Orange stage crude extract	+40.6	+33.6	−38	−29.8
Red stage crude extract	+29.8	+26	−43	−33.4
Purified oligomeric form	+60.2	+43.2	−21.8	−12.3
Purified monomeric form	−6.25	0	−51.5	−40.5
Mixed population form	+21.4	+27.8	−39.6	−32.1

Table 3. The kinetic properties of PFK preparations wth respect to F6P

Preparation	V_{max} (pkatals/ μg protein)	Hill coefficient (h)	$S_{0.5}$ (mM)
Green stage crude extract	0.2	0.7	6.5
Breaker stage crude extract	0.19	0.7	6.3
Orange stage crude extract	0.17	1.2	1.9
Red stage crude extract	0.17	1.25	2.3
Purified oligomeric form	62.3	0.72	5.8
Purified monomeric form	5.2	1	1.8
Mixed population form	1.7	1.26	2.2

ripening and the kinetic constants are summarized in Table 3. Figure 2(a) shows a double reciprocal plot of the kinetics of PFK from the green stage of tomato growth which shows the negatively cooperative interaction exhibited with F6P for the oligomeric form of the enzyme (Hill coefficient, $h = 0.7$). Figure 2(b) shows a double reciprocal plot of the kinetics of PFK from the orange stage of tomato growth which shows the positively cooperative interactions ($h > 1$) with F6P which is exhibited by the presence of a mixture of the monomeric and oligomeric forms of the enzyme. The monomeric form of the enzyme showed the simple Michaelis- Menten pattern of kinetics ($h = 1$) [11]. Changes in the kinetic properties with F6P for PFK preparations from various stages of tomato growth follow the changes associated with the dissociation of the oligomeric form of the enyzme. There is a slight decrease in V_{max} for PFK as the fruit enters the ripening stages. On the other hand, PFK from the preclimacteric stage exhibits negative cooperativity ($h = 0.7$) and low affinity to F6P (substrate concentration giving half maximal rate, $S_{0.5} = 6.3$–6.5 mM) while the enzyme from the post climacteric stage exhibits an apparent positive cooperativity ($h = 1.2$) and higher affinity to F6P ($S_{0.5} = 1.8$ 2.2 mM).

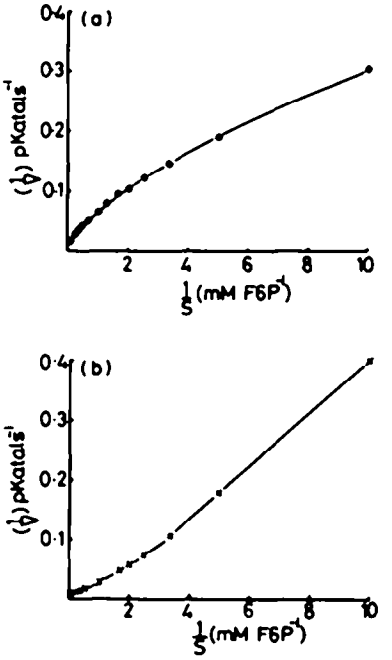

Fig. 2. A double reciprocal plot of the kinetics with F6P for PFK (a) from the green stage, (b) from the orange stage.

DISCUSSION

The *in vivo* activity of an enzyme is determined by factors which affect either the number of active enzyme molecules present (coarse control) or the degree to which the potential catalytic activity of these available enzyme molecules is expressed (fine control) [14]. There was no significant increase in the extractable activity of PFK during the various stages of ripening of tomato fruit and consequently, coarse control appears unlikely as a major factor regulating tomato PFK activity during the climacteric, which is in agreement with previous work on the

ripening of grapes [12] and bananas [2]. Nevertheless, the present work shows that there was a change in the molecular form of tomato PFK with ripening. The presence of PFK isoenzymes has been reported during the ripening of bananas [13] and tomatoes [14]. On the other hand, PFK from bananas was shown to remain in a single form but with different regulatory properties during ripening [2].

In addition to the changes in the molecular form of tomato PFK observed during ripening, the possibility of regulation of PFK activity by fine control will be discussed in relation to the available information on the concentrations of its substrates and modulators in the tomato fruit *in vivo*.

ATP concentration in the tomatoes (50–65 μM) decreases slightly at the climacteric stage and then rises until the red stage before it starts to decline again [4]. Mg^{2+} concentrations in the tomato range from 0.6 to 2 mM [15]. There is no evidence of the state in which ATP is present *in vivo*, but the concentrations of Mg^{2+} *in vivo* are sufficient to complex all the ATP. Regardless of this, the small changes in ATP are unlikely to account for the changes in PFK activity *in vivo* during the climacteric. The average cellular concentration of F6P was approximately 56 μM at the mature green stage. It declined during the climacteric period to 40 μM and then changed little [4]. Such variations in F6P concentration are again unlikely to have major effects on PFK activity.

Inorganic phosphate is the most potent activator for plant PFK [6, 13]. Pi concentration in tomatoes (2.2–3.5 mM) increases between the immature green and the breaker stage and then it remains constant throughout ripening [4]. For tomato PFK, Pi was shown to stimulate the activity of the oligomer and it promoted its dissociation [13]. Pi also relieved the inhibition of tomato PFK by PEP and citrate [13].

PEP is the most potent known inhibitor for plant PFK [5, 11]. The concentration of PEP in the tomatoes ranges from 13–30 μM being at its maximum in the immature green stage and it declines progressively with ripening [4]. Such a concentration could be effective in inhibiting the activity of the enzyme *in vivo* since the PEP concentration required to induce half maximal inhibition for the tomato enzyme was shown to be 9–10 μM [11] and this is within the physiological range. However, the apparent negative cooperativity with regard to F6P offers a major advantage in preventing the complete inhibition of PFK by PEP [11].

Plant PFK is generally less sensitive to inhibition by citrate than PFK from other sources [5, 6, 12]. For tomato PFK, the citrate concentration required for half maximal inhibition was 1.78 mM and this compares with the concentration of citrate in tomatoes which ranges from 10–20 mM [16]. In all cases, these concentrations are expressed as averages for entire tissues and may not reflect the concentrations of substrates and modulators at the PFK active site.

In conclusion, we would like to present a working hypothesis to explain the behaviour of PFK during the period of the climacteric in the tomato. This model envisages Pi and PEP being the major factors controlling PFK activity *in vivo* [13]. Leakage of Pi from the vacuole due to changes in membrane permeability as the climacteric is initiated [4, 17], leads to an increase in [Pi] in the cell compartment housing PFK and stimulates the activity of the enzyme by relieving the inhibition by PEP mainly but also possibly by citrate and the constraint imposed by the negative cooperativity with the substrate, F6P is removed. At the molecular level this effect involves dissociation of the oligomeric enzyme into subunits. In the later stages of ripening, it is envisaged that continuous leakage of citrate and Pi leads to the appearance of large quantities of the monomeric form. These monomers, which are more sensitive to inhibition by negative modulators and less sensitive to positive modulators than the oligomer [12] may be inhibited at the later stage of ripening and this may explain the decline in glycolytic rates following the climacteric peak. Many of the points of this hypothesis remain to be tested and the application of purely kinetic data is limited by the lack of knowledge of the situation *in vivo*. Little is known about the locus and environment of PFK *in vivo*, of the fluxes and local concentrations of its substrates and modulators.

EXPERIMENTAL

Plant materials. Tomatoes (*Lycopersicon esculentum* variety Eurocross BB) were grown in the IFR, Norwich Laboratory greenhouse. All of this work was done on fresh plant tissue. The tomatoes were selected by visual colour using a grading system of 1 for mature green tomatoes, 2 for breaker stage, 3 for 1/3 colour, 4 for 2/3 colour, 5 for pink, 6 for full red and 7 for overripe. In this variety, the climacteric peak is reached just beyond the breaker stage and respiration declines in the later stages of the ripening process.

Chemicals. Biochemicals were purchased from Boehringer, Mannheim and were of the best quality available. Ultrogel AcA 34 was from LKB, Sweden, and the protein determination reagents were the product of Biorad. Tris and dithioerythretol were from Sigma and all other chemicals were from B.D.H. Chemicals Ltd.

PFK extraction and assay. The extraction medium consisted of 0.2 M Tris–HCl, pH 7.5, containing 2 mM EDTA and 5 mM DTE. The tomatoes were cut and the outer wall of the pericarp together with the epidermis were taken. To each gram of plant tissue, 2 ml of the extraction medium and 50 mg polyclar AT were added. The mixture was then homogenized, filtered and centrifuged at 35 000 *g* for 25 min. The supernatant represents the crude extract which was dialysed against 5 mM Tris–HCl, pH 7.5, containing 2 mM EDTA and 2 mM DTE. The PFK activity was assayed by coupling the production of fructose 1,6-bisphosphate with the oxidation of NADH through aldolase, triosephosphate isomerase and α-glycerolphosphate dehydrogenase. The standard assay contained the following in a 1 ml cuvette: 0.16 μmol NADH, 5 μmol $MgCl_2$, 1 μmol ATP (Na salt), 2 μmol fructose 6-phosphate (Na salt), 0.4 units of α-glycerolphosphate dehydrogenase, 2 units of triosephosphate isomerase and 0.4 units of aldolase. For analysis of the kinetic data, the Hill equation was used in the form $v = V_{max}[S]^h/K_m + [S]^h$ where v = initial velocity; [S] = substrate concentration; K_m = substrate concentration giving half maximal rate; V_{max} = maximum velocity and h = Hill coefficient. A computer program based on an iteration procedure was used for estimating V_{max}, h and $S_{0.5}$ (substrate concentration giving half maximal rate) in relation to each other from a set of values of reaction rates and substrate concentrations. Details of these procedures were described elsewhere [10, 11].

M_r *determination.* The M_r of the enzyme was determined using gel permeation chromatography on Ultrogel AcA 34. The dialysed crude enzyme was first concentrated by adding solid polyethylene glycol 4000 up to a concentration of 65% (w/v).

Following centrifugation at 35000 *g* for 25 min, the precipitate was taken up in about 5 ml of 50 mM Tris–HCl, pH 7.5, containing 2 mM(EDTA and 5 mM DTE (buffer A). This preparation was then applied to an Ultrogel AcA 34 column (34 × 2.6 cm diameter) which was previously equilibrated with buffer A. The enzyme was eluted by the same buffer and fractions of 1 ml each were collected and assayed for PFK activity.

Protein determination. Proteins were determined by a dye binding assay [18] and this method was checked for its reliability against the method of Lowry *et al.* [19].

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