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IDENTIFICATION OF MULTIPLE FORMS OF PHOSPHOFRUCTOKINASE IN RIPENING DWARF CAVENDISH BANANA

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Key Word Index—*Musa sapientum*; Musaceae; banana; fruit ripening; phosphofructokinase; multiple forms; control of climacteric.

Abstract—The levels of six glycolytic intermediates and the activity of phosphofructokinase (PFK) were determined in Dwarf Cavendish banana at different stages of ripening between harvest and senescence. There was a 2.3-fold increase in the level of fructose-1,6-diphosphate between the preclimacteric and climacteric peak stage. The PFK preparations from preclimacteric and climacteric peak stages were purified *ca* 15-fold using Blue-Sepharose affinity chromatography. The electrophoretic studies with the enzyme preparations of these two stages of ripening indicated the presence of two forms of PFK at both stages of ripening.

INTRODUCTION

Ripeness indicates that the fruit is best for eating. There are distinct differences in the ripening phase of fruits that have a climacteric rise, e.g. banana, apples, mangoes [1]. In these fruits the respiratory climacteric is responsible for supplying the energy demands for the process of ripening [2]. Banana is a starchy fruit and the breakdown and metabolism of starch through the glycolytic pathway is the main event of ripening in this fruit. In the glycolytic pathway, the reaction catalysed by phosphofructokinase (PFK), [ATP: D-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11], is the first unique step and it represents a non-equilibrium reaction. It is known that the activity of plant PFK is affected by a number of metabolites like ATP, ADP, AMP and Pi [3–6]; citrate [3, 5]; phosphoenolpyruvate [5] and 6-phosphogluconate [7]. Earlier studies of the ripening of banana fruit have shown an increase in fructose-1,6-diphosphate (FDP) concentration parallel with the increase in climacteric respiration [8]. Later on, studies with banana in which the ripening was induced by ethylene showed that the increase in the activity of PFK was concomitant with the increase in FDP levels [9].

We have initiated the studies on PFK because of our interest in the regulation of carbohydrate metabolism in gamma-irradiated banana [10]. Gamma radiation induced a 7-day delay in the onset of climacteric. In order to understand the regulatory control on PFK by irradiation, one should fully know the regulatory mechanisms governing the levels of PFK activity in normal ripening banana. The present studies describe the partial purification of PFK by affinity chromatography, and identification of multiple forms of the enzyme.

RESULTS

In their studies Salminen and Young [9] used the bananas 11–14 days after harvest and the ripening was effected by the administration of ethylene. The main difference in the present study is that it was conducted with bananas of known maturity immediately after the harvest. The fruits were allowed to ripen normally without ethylene stimulation. The respiration studies with these fruits showed that the climacteric peak is obtained on the 15th day after harvest. The concentration of six glycolytic intermediates was determined at different stages of ripening (Table 1, Fig. 1). At the preclimacteric

Table 1. Changes in the levels of some glycolytic intermediates during the ripening of banana fruit

	nmol/g dry wt				
Glycolytic intermediates	day 0	6th day	12th day	15th day	19th day
Glucose-6-phosphate	340	354	411	614	200
Fructose-6-phosphate	243	235	201	427	91
Fructose-1,6-diphosphate	9	10	84	162	50
Triose phosphates	83	85	112	302	200
Phosphoenolpyruvate	154	163	230	507	253
Pyruvate	141	152	293	429	307

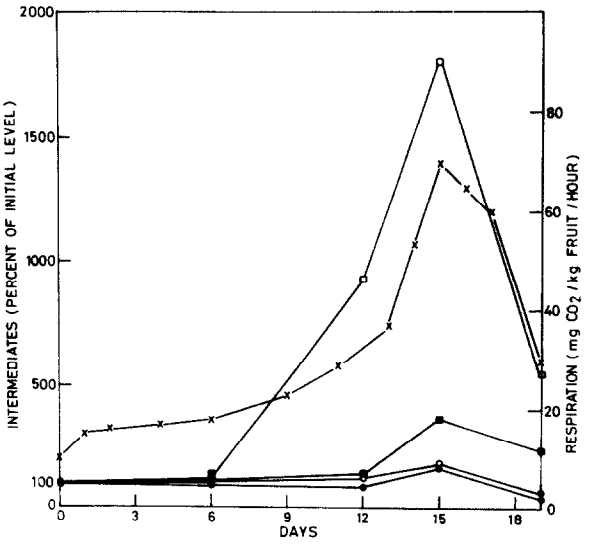

Fig. 1. Changes in the levels of G-6-P (○——○), F-6-P (●——●), FDP (□——□) and triose phosphates (■——■) in relation to respiration (×——×) of banana fruit. The initial levels are expressed in nmol/g dry wt and were: G-6-P, 340; F-6-P, 243; FDP, 9; TP, 83.

stage, i.e. up to the 6th day, the concentrations of all the intermediates did not change from the initial values. The concentration of FDP was lowest among the intermediates. With the climacteric upsurge, the concentration of FDP alone increased *ca* 18-fold on the 15th day, when the respiration rate was also at a maximum. So FDP is the only glycolytic intermediate which showed significant fluctuations during the ripening stages. In order to detect the regulatory sites in the pathway, determination of the crossover points based on Chance's theorem [11] was done. In Fig. 2, a crossover plot for the glycolytic intermediates between day 0 (immediately after harvest) and day 12 (climacteric rise) is given. The crossover point is between F-6-P and FDP from −0.19 to +1.6, which suggested the existence of a regulatory site at this step.

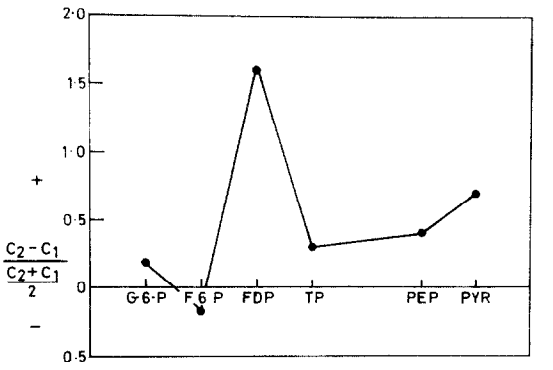

Fig. 2. Crossover plot for glycolytic intermediates between two stages of ripening; immediately after harvest (0 day) and at climacteric rise (12th day). C_1 is the concentration of the same intermediate at the 12th day after harvest.

Purification of PFK

We have seen that as the banana ripens there is a large increase in FDP content. This is an indication of PFK regulation. For the determination of the nature of this regulation during the climacteric rise, it was necessary to purify this enzyme from both preclimacteric and climacteric peak banana. There were not many reports on the purification of this enzyme from plant sources especially from fruits like banana, although partial purification has been obtained [9]. We have used precipitation of inactive proteins with polyethylene glycol, ammonium sulphate, heat treatment and finally affinity chromatography on Blue-Sepharose to obtain 16- and 14-fold purification of PFK from preclimacteric and climacteric tissues respectively (Table 2). The elution pattern of these two enzyme preparations from the Blue-Sepharose column by linear gradient of ATP is suggestive of the difference in the kinetic behaviour of the two proteins (Fig. 3). The climacteric enzyme required a lower concentration of ATP to elute it from the column. The preclimacteric enzyme was eluted at a higher concentration of ATP between fractions 18 and 27, while the climacteric enzyme eluted in fractions 11–20.

Table 2. Purification of phosphofructokinase

Stage of ripening	Fractionation step	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	% recovery
Preclimacteric preparation	1. Crude extract	112.0	2530	22.5	100
	2. 8% PEG treatment	85.1	2440	28.7	97
	3. $(NH_4)_2SO_4$ precipitation (20–50%)	28.1	1990	71.0	79
	4. Heat treatment	15.2	1650	109.0	65
	5. Affinity chromatography (fractions 18–27 pooled)	2.5	880	353.0	35
Climacteric peak preparation	1. Crude extract	136.0	5980	44.1	100
	2. 8% PEG treatment	106.0	5621	52.9	94
	3. $(NH_4)_2SO_4$ precipitation (20–50%)	42.6	4310	101.0	72
	4. Heat treatment	19.7	3650	185.0	61
	5. Affinity chromatography (fractions 11–20 pooled)	2.9	1760	606.0	29

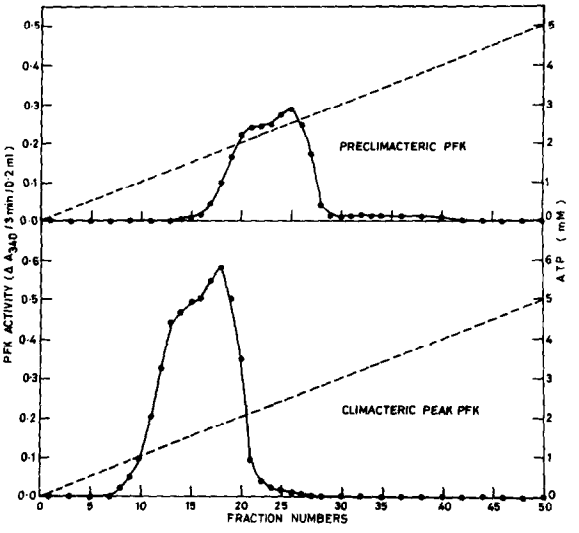

Fig. 3. Blue-Sepharose chromatography of PFK isolated from preclimacteric and climacteric peak banana. PFK activity (●——●) and a linear gradient (----) of ATP (0–5 mM).

Polyacrylamide gel electrophoresis

Regulatory enzymes are known to occur in oligomeric forms and activation is often recognized with a change in subunit structure. Hence, an attempt was made to compare the migration velocities of the enzyme from both stages of ripening. The proteins of the enzyme preparations before purification by affinity chromatography were separated on polyacrylamide gel. Since the determination of enzyme activity with formazan formation gave false bands, the analysis of the activity was done in 2 mm transverse slices of the gel. Each slice was incubated in a reaction medium with and without F-6-P. The amount of FDP formed in each medium was determined with auxiliary enzymes and NADH, and the results are shown in Fig. 4. The largest amount of FDP formation was seen with slices 15–18, 3 cm from the origin. The amount of protein applied on the gels was the same in both cases. It is clear from the histograms that the activity of climacteric PFK is higher in comparison with preclimacteric enzyme. The distribution of the enzyme activity in both stages of ripening gave an indication of the possible presence of multiple forms, although it was not a conclusive demonstration. Electrophoresis was then performed with purified preparations obtained by affinity chromatography. In this case the assay of the activity was done directly on the gel by using a formazan-formation technique (Fig. 5). Two distinct bands were visible *ca* 3 cm from the origin, suggesting the presence of two forms of PFK in both stages of ripening. The two bands were sliced from the unstained gels corresponding to the stained gels and found to have PFK activity. For convenience of detection, the amount of protein applied on the gel from the climacteric preparation was only half, compared to the preclimacteric preparation. Thus the results indicate the presence of two multiple forms of PFK in banana at both stages of ripening.

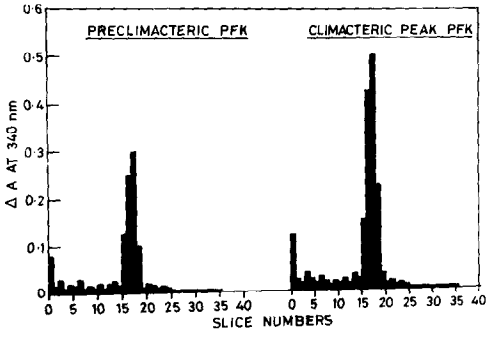

Fig. 4. Histogram of PFK activity of preclimacteric and climacteric peak fruit as determined with 2 mm slices of polyacrylamide gel.

DISCUSSION

In banana, the increase in the climacteric respiration is closely associated with the increase in the level of a key glycolytic intermediate, FDP. This finding supports the observed activation of PFK at the onset of climacteric. In ripening tomato fruit, Chalmers and Rowan [12] have also observed the activation of PFK by the release of inorganic phosphate from vacuoles due to permeability changes. The increase in the activity of PFK at the upsurge of the climacteric appears to be due to the changes in the amount and availability of enzymic effectors altering the kinetic properties, rather than to a net increase in enzyme *per se*. One of the major studies on banana PFK conducted by Salminen and Young on artificially ripened fruit also indicated this possibility. In our studies the bananas were taken from the plant

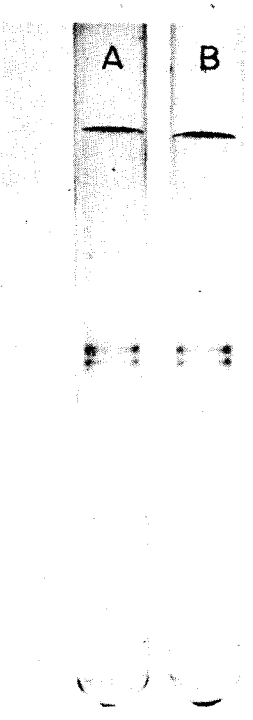

Fig. 5. Polyacrylamide gel electrophoresis of partially purified PFK preparations obtained by affinity chromatography. A, preclimacteric preparation (129 μg) and B, climacteric peak preparation (63 μg). Migration from top to bottom. A faint band of marker dye (bromophenol blue) can be seen at the bottom portions of each gel.

immediately after harvest and were allowed to ripen under normal conditions. The changes in the levels of glycolytic intermediates (Table 1) agreed well with the observations of Barker and Solomos [8] in Lactan variety and Salminen and Young [13] in Valery bananas.

The PFK activity from climacteric fruit is always higher than that of preclimacteric fruit at all stages of purification (Table 2). In order to eliminate the possibility that the increased activity of PFK at the climacteric peak is due to the increased extractability of the enzyme from the softened pulp tissue, we have compared the extractability of two other enzymes at different stages of ripening. The results in Fig. 6 showed that the other enzymes showed only marginal increase. If the increased activity is due to increased extractability, it would have affected all three enzymes in the same way. Other alternatives for the increase in the PFK activity levels are (1) increased synthesis of enzyme protein or formation of new isozymes, (2) the activation of the existing enzyme through the changes in its regulatory properties. In our studies, apart from the pre-existing two forms, we find no evidence for the synthesis of new isoenzymes. The evidence presented in this paper tends to support the idea that there may be an alteration in the regenerating properties of the enzyme.

We have not conducted kinetic studies with the purified enzyme because of the presence of two isoenzymes which may complicate the substrate saturation studies. First of all, our aim is to separate the two multiple forms with good recovery of activity and to study the kinetic and regulatory properties. Such a study will help to throw more light on the regulation of PFK during the climacteric in ripening banana. Multiple forms of PFK have been reported from animals [14, 15], plants [16, 17] and bacteria [18, 19]. Spinach leaf is reported to contain two PFK activities with distinct properties. One of the activities had chloroplastic origin, while the other was of cytoplasmic origin [16]. Of the two PFK activities shown in endosperm of developing castor beans, one was attributed to the proplastids [17]. The studies by Salminen and Young [9] did not show the presence of two forms of PFK in banana. This may be due to the fact that in the polyacrylamide gel analysis, the gel slices taken for assay were too big (5 mm) to allow for the detection of multiple forms. There is a possibility of multiple forms being assayed in a single slice. At present the exact role of two multiple forms of PFK and their activation remains to be explained. It is open to speculation whether these multiple forms observed represent a group of different enzymes with similar functions, or multiple forms of the same enzyme which differ from each other in subunit composition. We have undertaken studies on the separation and characterization of these four proteins for further understanding of the regulatory properties of PFK.

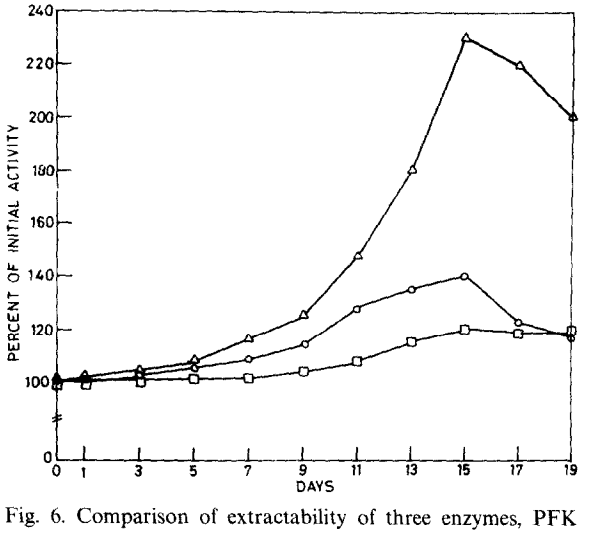

Fig. 6. Comparison of extractability of three enzymes, PFK (△——△), aldolase (□——□) and 6-phosphogluconate dehydrogenase (○——○) at different stages of ripening. The initial levels are expressed in mU/g dry wt and were: PFK, 312; aldolase, 902; and 6-PG dehydrogenase, 937.

EXPERIMENTAL

Chemicals. All substrates, cofactors and polyethylene glycol (PEG-4000) were obtained from Sigma. Auxiliary enzymes were supplied by Boehringer. Blue-Sepharose CL-6B was the product of Pharmacia. All other reagents used were of analytical grade.

Banana. The variety used in this study was Dwarf Cavendish (*Musa cavendishii*), obtained from the orchard within 8 hr of harvest. At harvest, the fruits were 70% mature as assessed by the pulp to skin ratio [20]. Fruits were stored in perforated polyethylene bags at 20° and 85% relative humidity. Fruits were taken for analysis every alternate day in the preclimacteric stage, and daily from the beginning of the climacteric rise to postclimacteric stage. The respiration of the fruits was monitored as described in our earlier studies [10]. After monitoring the respiration, the same fruits were peeled and cut into 2 mm slices. These slices were frozen in liquid N_2 and lyophilized. The freeze-dried slices were ground to a fine powder in a mortar with pestle and stored at −30°.

Extraction of enzyme. Lyophilized powder (1 g) obtained from each stage of ripening was extracted with 10 ml of a medium consisting of 50 mM Tris–HCl (pH 8.5), 1 mM EDTA, 5 mM $MgCl_2$, 20 mM 2-mercaptoethanol and 0.2% Triton X-100, in a mortar and pestle precooled with liquid N_2. After the addition of the extraction medium, the whole mass was frozen with liquid N_2, and thawed by grinding. The crude homogenate was directly centrifuged at 48 000 g for 20 min at 0°. The supernatant from the centrifugation was used as an enzyme prep. to assay the activities of PFK, aldolase and 6-phosphogluconate dehydrogenase.

Assay of enzyme activities. All three enzymes were assayed spectrophotometrically at 24° by following the change in A at 340 nm. The total vol. of the assay mixture was 3 ml. The reaction was initiated by the addition of enzyme prep. and the assays were made on the linear range with respect to time and enzyme concn. The assay medium for PFK contained 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 10 mM fructose-6-phosphate (F-6-P), 1 mM ATP, 0.15 mM NADH, 0.5 U aldolase, 90 U triosephosphate isomerase and 1.0 U α-glycerophosphate dehydrogenase. The assay medium for aldolase was identical with that for PFK except F-6-P, ATP and aldolase were omitted and 5 mM fructose-1,6-diphosphate was included. The assay medium for 6-phosphogluconate dehydrogenase contained 50 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 0.16 mM $NADP^+$ and 5 mM 6-phosphogluconate. All enzyme activities were expressed in milliunits (mU). One mU of enzyme is defined as the amount of the enzyme that catalyses the formation of 1 nmol of product per min, under the conditions of the assay described above. Sp. act. was expressed as mU/mg protein. Protein was estimated by a modified method of Lowry as described in ref. [21].

Estimation of glycolytic intermediates. Banana pulp samples (3 g) in duplicate were taken from different stages of ripening namely, immediately after harvest (0 day); preclimacteric stage (6th day); climacteric rise (12th day); climacteric peak (15th day) and postclimacteric stage (19th day). The medium for extraction was the same as that used for the enzyme extraction. The sample from each stage was homogenized in a mortar in 30 ml of the extraction medium containing 0.66 N $HClO_4$. The homogenate was centrifuged at 48 000 g at 0° for 30 min and the supernatant was stored in an ice bath. The residue was resuspended in 7 ml of an ice-cold extraction medium and recentrifuged. The supernatant fluids were combined and the pH was adjusted to 7.2 with 5 M K_2CO_3 soln. The soln was allowed to stand in an ice bath for 20 min and then centrifuged at 40 000 g for 10 min and the supernatant was used to determine the levels of G-6-P, F-6-P, triose phosphates (TP), phosphoenolpyruvate (PEP) and pyruvate according to the method of ref. [22].

Purification of phosphofructokinase. Purification of PFK was attempted from two stages; preclimacteric stage (preclimacteric enzyme) and climacteric peak stage (climacteric enzyme)

Step 1, crude extract: From each stage, 10 g of lyophilized tissue was homogenized with 100 ml of extraction medium and centrifuged at 48 000 g for 20 min. All operations were performed at 0–4° unless otherwise indicated.

Step 2, polyethylene glycol treatment: Solid polyethylene glycol 4000, was slowly added to the 48 000 g supernatant to a final concn of 8%. The soln was stirred slowly with a magnetic stirrer for 15 min and then centrifuged at 40 000 g for 20 min. The ppt. was discarded.

Step 3, $(NH_4)_2SO_4$ fractionation: Crystalline $(NH_4)_2SO_4$ was slowly added to the PEG-treated supernatant to 20% satn. The pH of the soln was adjusted to 7.2. The soln was stirred slowly for 10 min and then centrifuged at 48 000 g for 20 min. To the 20% satd supernatant fluid sufficient $(NH_4)_2SO_4$ was added to bring the soln to 50% satn. Adjusting the pH, stirring and centrifugation were repeated. The ppt. from 20 to 50% satn was dissolved in 10 ml of medium consisting of 10 mM Tris–HCl, pH 7.4, 5 mM dithiothreitol and 0.5 mM ATP and centrifuged at 40 000 g for 20 min.

Step 4, heat treatment: The supernatant soln from the 20 to 50% fraction was heated for 3 min in a 60° bath, chilled to 0° immediately at the end of the period and centrifuged for 20 min at 30 000 g.

Step 5, affinity chromatography: The supernatant soln from the heat treatment step was applied on a column (bed vol. 10 ml) filled with Cibacron Blue-Sepharose equilibrated with 50 mM Tris–HCl buffer, pH 7.4, supplemented with 10 mM 2-mercaptoethanol (starting buffer). After the adsorption of the enzyme, the column was washed with a 10-fold vol. of the starting buffer. A linear gradient of ATP (0–5 mM in starting buffer) was then employed to elute the PFK. The fraction vol. was 1.0 ml and the flow rate was 14.5 ml/hr. The fractions having a PFK activity more than 40 mU were pooled.

Polyacrylamide gel electrophoresis. The proteins of the partially purified PFK prep. obtained from the Blue-Sepharose chromatography were separated on 5% polyacrylamide gel as described in ref. [23]. Cylindrical gels 8 cm long were prepared by mixing the solns A, B and H_2O in the ratio 2:1:3. Electrophoresis was carried out at 1° for 3 hr at a constant current of 3 mA/gel. After the electrophoresis, the PFK activity on the gels was located by formazan formation according to the procedure described in ref. [23].

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