Phytochemistry, 1972, Vol 11, pp 119 to 125 Pergamon Press Printed in England

PROPERTIES OF ACIDIC AND ALKALINE FRUCTOSE-1,6-DIPHOSPHATASE FROM γ-IRRADIATED BANANA

K. K. SURENDRANATHAN and P. MADHUSUDANAN NAIR

Biochemistry and Food Technology Division, Bhabha Atomic Research Centre, Trombay, Bombay-85, India

(*Received* 20 *April* 1971)

Abstract—Irradiation of preclimacteric Dwarf Cavendish bananas at 35 krad caused a gradual activation of fructose-1,6-diphosphatase, which reached a maximal 5-fold increase in activity in 3 days. Two enzymic components showing activity in acid (pH 6 6) and alkali (pH 8 8) were separated by calcium phosphate gel adsorption and elution Both the enzymes required Mg^{2+} ions for activity The catalytic properties of acidic and alkaline FDPase were quite different The alkaline enzyme was more sensitive towards the concentration of substrate, and allosteric inhibitor AMP Activation by disulfide reagent and EDTA was observed only in the case of alkaline enzyme

INTRODUCTION

IN BANANAS, there is a rapid change in carbohydrate metabolism during ripening Tager and Biale[1] have reported a shift from the pentose phosphate pathway to the glycolytic pathway of carbohydrate dissimilation in ripening bananas The most significant change observed, was the increase in the concentration of fructose-1,6-diphosphate, which closely followed the climacteric rise in respiration, as a result of activation of phosphofructokinase [2] In our laboratory, Thomas *et al* [3] have successfully demonstrated a delay in ripening of Dwarf Cavendish banana, when they were subjected to γ-irradiation at preclimacteric state. Since it was established that a close relationship exists between ripening and fructose-1,6-diphosphate content in banana, we examined the status of fructose-1,6-diphosphatase (FDPase) in unirradiated and irradiated banana It was observed that γ-irradiation caused an increase in FDPase activity in Dwarf Cavendish bananas. We now describe the purification, separation and some properties of banana FDPases

RESULTS

Effect of γ-Irradiation on FDPase Activity in Banana

Unirradiated, fresh, 80% mature Dwarf Cavendish banana showed two FDPase activities, one of which had a maximum activity at acidic pH, and the other at alkaline pH, although these activities were quite low There was no change in these activities during seven days storage after harvest But 35 krad γ-irradiation, which delayed the onset of climacteric for about 4 days, caused an appreciable and very consistent increase in these two activities within 3 days of irradiation. On the third day, both activities showed 5-fold increase compared to those of an unirradiated sample On further storage, there was a slight decrease in these activities, even though on the seventh day these activities were considerably more than

[1] J M TAGER and J B BIALE, *Physiol Plant* **10**, 79 (1957).
[2] J BARKER and T SOLOMAS, *Nature, Lond* **196**, 189 (1962)
[3] P THOMAS, S D DHARKAR and A SREENIVASAN, *J Food Sci* **36**, 243 (1971).

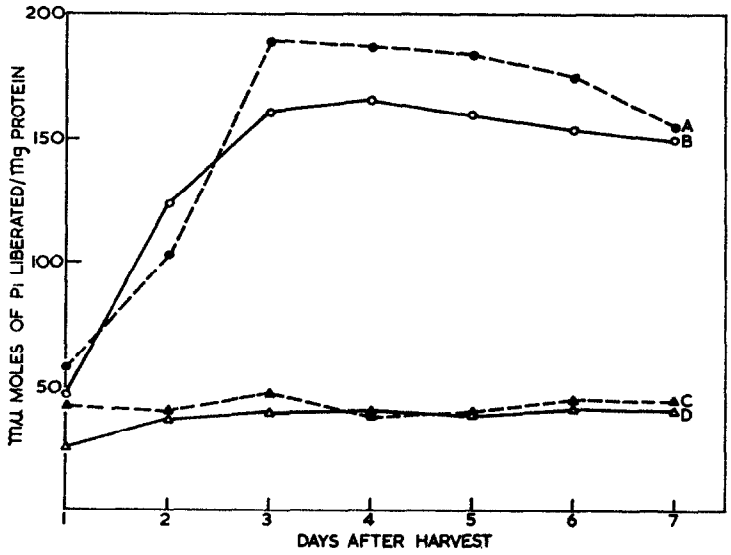

FIG 1 EFFECT OF γ-IRRADIATION ON THE ACTIVATION OF FRUCTOSE-1,6-DIPHOSPHATASE IN DWARF CAVENDISH BANANA.

Specific activity of acidic (pH 6 6) and alkaline (pH 8 8) FDPase after irradiation of Dwarf Cavendish banana at preclimacteric state, was determined at different time intervals Crude homogenate was used as enzyme preparation Protein concentration in the reaction mixture in these experiments varied from 1 to 1 5 mg Curves (A) acidic enzyme irradiated, (B) alkaline enzyme, irradiated, (C) acidic enzyme, control, (D) alkaline control

that of unirradiated sample (Fig 1) A perusal of the data on pH dependance clearly indicates that unfractionated enzyme showed two pH optima for FDPase activity This result indicates the presence of two enzyme components

Separation of Acidic and Alkaline FDPases

The presence of two different fractions in the crude enzyme preparation was further established by their separation on purification Typical purification data are presented in Table 1 Calcium phosphate gel adsorbed the alkaline enzyme preferentially under the conditions employed. pH adjustment in the purification procedure is very critical, in the sense that no adsorption was observed at pH 6·0 or above At pH's below 5 0, the alkaline enzyme was completely precipitated and inactivated By following the procedure described

TABLE 1. PROGRESS OF PURIFICATION OF FDPase FROM γ-IRRADIATED DWARF CAVENDISH BANANA

Fraction	Volume (ml)	Protein (μg/ml)	Specific activity		Total activity	
			Acidic	Alkaline	Acidic	Alkaline
Crude homogenate	100	280	857	714	24,000	20,000
$(NH_4)_2SO_4$ fraction	40	68	2941	2500	8000	6800
Calcium phosphate gel supernatant	35	7	20,730	0	7000	0
Calcium phosphate gel eluate	20	8	0	12,320	0	3400

above, a 20-fold purification of both enzymes with about 20% recovery of total activity can be achieved The purified enzyme was unstable. Storage even at −30° for 1 week caused almost complete inactivation of alkaline phosphatase and 60% inactivation of acidic phosphatase

pH Optimum

The effect of pH on the activity of both the purified preparations was tested at pH ranges from 5 4 to 10 and this confirmed the presence of two distinct, well seperable FDPase in banana. The optimum pH for acidic and alkaline FDPases was 6 6 and 8 8 respectively

Stoichiometry and Substrate Requirements for the Reaction

The two activities isolated from banana are established as C-1 fructose-1,6-diphosphatase by converting the product of the reaction to G-6-P with phosphoglucoisomerase, and estimating G-6-P using G-6-P dehydrogenase and NADP. A strict one to one mole stoichiometry between Pi liberated and F-6-P formed was obtained in both cases.

The enzyme was specific for fructose-1,6-diphosphate. It did not hydrolyse F-6-P, F-1-P, G-6-P, G-1-P and β-glycerophosphate or pyrophosphate with Pi assay system. The substrate FDP has a pronounced effect on the enzyme activity Maximum activity was obtained for both FDPases at a substrate concentration of 0·2 mM. Higher substrate concentrations inhibited both activities, although alkaline phosphatase was more susceptible to higher concentration of substrate (Fig 2).

Metal Requirement

Mg^{2+} ions were essential for the activity. The omission of Mg^{2+} from the reaction mixture abolished enzyme activity Both the enzyme activities responded to the same extent to the concentration of Mg^{2+} present in the reaction mixture. Optimum Mg^{2+} requirement was 20 μmoles in both cases. These enzymes showed a preference for Mg^{2+} ions Mn^{2+} ions were not as effective as Mg^{2+} in activating the reaction Even at 40 μmoles Mn^{2+} showed only about 60% of the activity achieved by Mg^{2+} ions

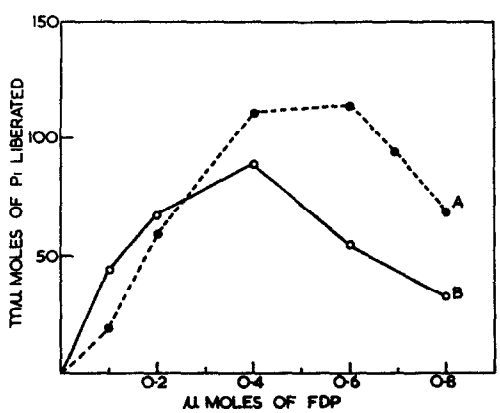

FIG 2. EFFECT OF FRUCTOSE 1,6-DIPHOSPHATE CONCENTRATION ON THE ACTIVITY OF ACIDIC AND ALKALINE FDPASE FROM DWARF CAVENDISH BANANA

Experimental details are same as given under Experimental except different concentrations of F 1,6-diphosphate as mentioned in the figures are included in the reaction mixture. Curves (A) acidic FDPase, (B) alkaline FDPase

TABLE 2 EFFECT OF DIFFERENT CONCENTRATION OF AMP ON ACIDIC AND ALKALINE FDPase

Concentration of AMP (M)	Per cent inhibition	
	Acidic enzyme	Alkaline enzyme
1×10^{-6}	12 5	33 5
5×10^{-6}	25	50 0
1×10^{-5}	37	63 5
5×10^{-5}	50	74 0
1×10^{-4}	75	98 0

The enzyme was preincubated for 2 min with different concentration of AMP before the addition of substrate A control without AMP was also done parallel with this experiment

Effect of AMP, EDTA and Disulfide Reagents

In recent years the function of FDPase as a regulatory enzyme in gluconeogenesis in animal and microbial system is well established.[4–8] FDPase is an allosteric enzyme and AMP functions as the allosteric effector In photosynthetic tissue it can be regulated by ferredoxin also [9] Most of the purified preparations of FDPase showed little activity in the physiological pH range Pontremoli *et al* [10] have reported that various sulfhydryl reagents can stimulate the activity of liver FDPase at neutral pH Treatment of the enzyme with EDTA also brought about an additional peak of activity in the case of *Dictyostelium discoidum* FDPase.[11] In order to determine the catalytic nature of acidic and alkaline FDPases from banana, the effect of the above reagents on enzyme activity was examined The results

TABLE 3 EFFECT OF SULFHYDRYL REAGENTS AND EDTA ON ACIDIC AND ALKALINE FDPase

Additions	Relative activity	
	Acidic	Alkaline
None	100	100
Cystamine	102	374
Ethylenediamine tetraacetate (EDTA)	114	284
55′-Dithiobis-(2 nitro benzoic acid) (DTNB)	92	152

These compounds were added at 0 2 μmoles/ml concentration the standard assay mixture Enzyme in this experiment was preincubated for 2 min with the activation before the addition of substrate

[4] D G FRAENKAL, S PONTREMOLI and B L HORECKER, *Arch Biochem Biophys* **114**, 4 (1966)
[5] H A KREBS and M WOODFORD, *Biochem J* **94**, 436 (1965)
[6] O M ROSEN, *Arch Biochem Biophys* **114**, 31 (1966)
[7] O M ROSEN, S M ROSEN and B L HORECKER, *Arch Biochem Biophys* **112**, 411 (1965)
[8] K TAKETA and B M POGELL, *J Biol Chem* **240**, 651 (1965)
[9] B B BUCHANAN, P P KABERER and D I ARNON, *Biochem Biophys Res Commun* **29**, 74 (1967)
[10] S PONTREMOLI, S TARANIELLO, N ENSER, S SHAPIRO and B L HORECKER, *Proc Natl Acad Sci U S* **55**, 286 (1967)
[11] P BAUMANN and B T WRIGHT, *Biochemistry* **8**, 1655 (1969)

TABLE 4. ACTIVITY OF ALKALINE FDPase AT DIFFERENT pH VALUES IN THE PRESENCE OF CYSTAMINE OR EDTA

pH	Activity in mμmoles of Pi liberated EDTA	Cystamine
5 4	0	0
5 8	0	0
6 2	0	0
6 6	10	22
7 05	15	33
7 5	22	45
8 2	55	108
8 6	112	170
8 8	178	210
9 2	100	158
9 4	49	64

The enzyme was preincubated at various pHs either with 0 2 μmoles/ml of EDTA or cystamine for 2 min before the addition of substrate

(Table 2) show that both the banana FDPases were sensitive to AMP concentrations, but the degree of inhibition is greater with alkaline phosphatase AMP, as low as 10^{-6} M, inhibited alkaline phosphatase to 33% whereas acidic phosphatase was inhibited by about only 12 5%. AMP (10^{-4} M) inhibited the alkaline enzyme completely At this concentration AMP exhibited only 75% inhibition with acidic enzyme. Studies with disulfide reagents showed (Table 3) that only the alkaline enzyme activity was stimulated by these reagents. The effect of EDTA was also similar to that of sulfhydryl reagents. But these reagents have failed to induce the activity at neutral pH for the alkaline enzyme, although there was about 2-fold increase in activity at pH 8 8 (Table 4).

DISCUSSION

FDPase isolated from banana after γ-irradiation showed certain distinct and different characteristics from other FDPases reported from plant and animal sources. The fact that this enzyme is dormant in unirradiated banana, and can be activated by γ-irradiation suggests that this enzyme plays a regulatory role in the banana. One of the factors which is known to regulate the activity of rabbit liver FDPase, is the action of disulfide reagents on the enzyme protein, which modify one or more sulfhydryl groups [12] The enzyme thus activated exhibited activity at neutral pH, which was absent in the native enzyme Since gamma irradiation is known to alter the sulfhydryl groups of proteins this activation of banana FDPase with γ-irradiation may be due to the modification of sulfhydryl groups of the enzyme protein with corresponding conformational changes. Little *et al.*[13] have reported that X-rays modify the catalytic activity of rabbit liver FDPase. The dose response on *in vitro* irradiation of the enzyme with X-rays showed an initial activation, followed by subsequent exponential inactivation The loss in activity can be partially reversed by treating the exposed enzyme with cystamine. These observations also suggest a possible function of sulfhydryl groups in radiation induced stimulation of FDPase. Apart from this report, there is no other information available on the effect of γ-irradiation or X-rays on FDPase Our finding is novel in

[12] C LITTLE, T. SANNER and A PHIL, *Europ J Biochem* **8**, 229 (1969)
[13] C LITTLE, T SANNER and A. PHIL, *Biochim Biophys Acta* **178**, 83 (1969)

the sense that the activation of banana FDPase occurred only on *in vivo* irradiation Irradiation of the crude enzyme isolated from unirradiated banana to 35 krad caused complete inactivation of both enzyme activities The slow and gradual activation of these activities on irradiation of the banana fruit may be due to delay in the alteration of sulfhydryl groups Recently Scala *et al* [14] have suggested that, in germinating castor beans, gibberellic acid induced the *de novo* synthesis of a neutral FDPase Hence the possibility of a *de novo* synthesis of these enzyme proteins induced by irradiation cannot be excluded

Radiation induced FDPase showed two pH optima and the enzyme components responsible for this can be resolved Their responses to substrate, AMP and disulfide reagents differed considerably. These differences indicate that they have altogether different catalytic properties Another important difference of banana alkaline FDPase from other known FDPases, like rabbit liver enzyme or *Dictyostelium discodium* enzyme, is its failure to induce activity at neutral pH on treatment with either cystamine or EDTA (Table 4)

EXPERIMENTAL

Banana used in this study was Dwarf Cavendish (*Musa cavendishii*) variety Fruits were harvested and fingers of known maturity were irradiated on the same day after the latex flow had ceased Irradiation was carried out in a gamma cell 220 in air at 25°, at a dose rate of 2 76 krad/min The fruits received 35 krad dose

Assay of FDPase activity The enzyme assay with the crude extract was carried out at pH 6 6 (0 05 M sodium maleate buffer) and 8 8 (0 05 M tris-HCl buffer). The reaction mixture consisted of 25 μmoles buffer, 20 μmoles $MgCl_2$, 0 4 μmoles FDP and appropriate amount of enzyme (150 μg protein for the crude enzyme and 3 μg for purified preparation) in a total vol of 2 ml The reaction mixture was incubated for 20 min at 37°

The product of the reaction was determined either by estimating Pi liberated[15] after stopping the reaction with 10% TCA, or by using the spectrophotometric method[16] which estimates the fructose-6-phosphate formed Spectrophotometric assay was carried out as follows 0 7 ml of FDPase assay mixture was added to a reaction mixture in a cuvette containing 100 μmoles tris-HCl buffer pH 7 5, 10 μmoles $MgCl_2$, 1 75 μmoles EDTA, 12 mg of mung bean phosphoglucoisomerase, prepared by the method of Ramasarma and Giri,[17] 25 μg of glucose-6-phosphate dehydrogenase This mixture was incubated for 5 min at room temp and 0 2 μmoles of NADP was added and change in absorbancy at 340 nm was measured

For routine assay Pi liberated was estimated A unit of enzyme activity was defined as the amount of enzyme which liberates 1 μmole of Pi under standard assay conditions Specific activity refers to units per milligram of protein Protein determinations were carried out according to the Lowry method [18]

Purification of FDPase 35 krad irradiated banana were stored at 20° and RH 85–90% for 3 days to get maximum activation of FDPase Samples were then deskinned, the pulp tissue was cut into small pieces and quickly frozen with liquid N_2 The frozen samples were stored at −30° and used as the source of the enzyme

Acetone powder preparation The frozen banana was blended in a precooled Waring blender with five times its weight of cold acetone (−30°) for 1 min at 0–4° The slurry was filtered through a Buchner funnel and the residue was again blended with half the original amount of cold acetone and filtered again This process was repeated once more The powder was dried at 0–4° and stored at −30° All operations hereafter were carried out at 0–4°

35 g of acetone powder was mixed with 125 ml of 0 1 M tris-buffer pH 7 5 in a precooled mortar and pestle This mixture was allowed to freeze at −30° and thawed out by grinding During this process the temperature of the mixture was not allowed to exceed 5° The ground mass was filtered through a double layer of cheese cloth and the filtrate was centrifuged at 20,000 *g* for 20 min The supernatant was used as the crude enzyme preparation

To 100 ml of crude homogenate 17 6 g of solid $(NH_4)_2SO_4$ was dissolved with stirring The solution was held for 10 min for complete precipitation The precipitate formed was collected by centrifuging at 20,000 *g* for 20 min The residue was dissolved in 40 ml 0 1 M tris-buffer pH 7 5 Dialysis for 4 hr resulted in considerable inactivation of both FDPase activities, so this step was omitted The pH of 35 ml $(NH_4)_2SO_4$

[14] J SCALA, C PATRICK and G MACBETH, *Phytochem* **8**, 37 (1969)
[15] C H FISKE and Y SUBBARROW, *J Biol Chem* **66**, 375 (1925)
[16] E RACKER, *in Methods in Enzymology* (edited by S P COLOWICK and N O KAPLAN), Vol 5, p 272, Academic Press, New York (1963)
[17] T RAMASARMA and K V GIRI, *Arch Biochem Biophys* **62**, 91 (1956)
[18] O H LOWRY, N J ROSEBROUGH, A L FARR and R J RANDALL, *J Biol Chem* **193**, 265 (1959)

fraction was adjusted to 5 4 with dilute HOAc and the solution was treated with calcium phosphate gel in protein to gel ratio (1 7) The mixture was stirred for 30 min for complete adsorption of alkaline phosphatase On centrifuging this slurry for 10 min at 10,000 *g*, the supernatant obtained contained all acidic phosphatase activity The residue was uniformly suspended in 20 ml 0 1 M tris-buffer pH 7 5 and the mixture was kept with stirring for 30 min for complete elution The supernatant obtained on centrifuging the mixture showed only alkaline phosphatase activity

Acknowledgements—The authors thank Dr A Sreenivasen for his helpful suggestions and criticisms

Key Word Index—Musa cavendishii, Musaceae, banana, fructose-1,6-diphosphatase, γ-irradiation