Phytochemistry, 1976, Vol. 15, pp. 1437–1439. Pergamon Press. Printed in England.

FRUCTOSE-1,6-DIPHOSPHATASE FROM *MANGIFERA INDICA*

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(*Revised received* 22 *March* 1976)

Key Word Index—*Mangifera indica*; mangofruit; fructose-1,6-diphosphatase; properties and regulation.

Abstract—Fructose-1,6-diphosphatase (FDPase) from unripe mango was separated into two components by ammonium sulfate fractionation, one active at pH 6 (acidic FDPase) and the other at pH 8.5 (alkaline FDPase). The alkaline component had a lower K_m (0.15×10^{-3} M) than the acidic component (1.7×10^{-3} M) towards the substrate (FDP) and the allosteric inhibitor AMP. It also showed greater heat stability and higher activation in the presence of EDTA as compared to the acidic FDPase. Both components showed a higher activation with Mn^{2+} ions than with Mg^{2+} ions.

INTRODUCTION

Fructose-1,6-diphosphatase (EC 3.1.3.11) is an allosteric regulatory enzyme of gluconeogenesis [1] and it catalyses the hydrolysis of fructose-1,6-diphosphate to form fructose-6-P and Pi. In addition to control of synthesis and activity [2], rapid loss of activity was observed when glucose was added to a yeast culture exhibiting high levels of FDPase. Here, the observed inactivation of the enzyme was attributed to a proteolytic process [3,4]. FDPase is also involved in the "futile cycle" with phosphofructokinase, to regulate the hydrolysis of ATP in animal tissues [5–7]. The net result of these two enzymic reactions is the hydrolysis of ATP as shown below:

$$\text{Mg ATP} + \text{Fructose-6-P} \rightarrow \text{Fructose-1,6-P2} + \text{MgADP} \quad (1)$$

$$\text{Fructose-1,6-P}_2 \rightarrow \text{Fructose-6-P} + \text{Pi} \quad (2)$$

Thus, in a cell where both enzymes are present, regulation of the activities of these two enzymes is needed to prevent waste of energy. This is probably achieved by the presence of AMP which shows reciprocal activator–inhibitor relationship with these enzymes. FDPase is inhibited by AMP [8–11] and activated by PEP [12]. Pogell *et al.* [13] have shown that the activity of crude FDPase is stimulated by pure muscle phosphofructokinase.

Two enzymic components of FDPase showing activity in acid (pH 6.6) and alkali (pH 8.8) were demonstrated in irradiated preclimacteric dwarf Cavendish banana [14]. These acidic and alkaline FDPases were quite different with regard to sensitivity towards concentration of FDP, activation by disulfide reagents and EDTA, and to allosteric inhibition by AMP. In this paper we describe some of the properties of FDPase of mango.

RESULTS AND DISCUSSION

Levels of FDPase in ripening mango

Mature, unripe mango showed two peaks for FDPase activity, one at pH 6 and the other at pH 8.5, the activity of the alkaline fraction being quite low. It was observed that the activity of the acidic FDPase was maximum in the unripe stage of the fruit (3.0 μmol Pi/mg protein/hr), diminished (× 0.74) during the early stage of ripening and then increased (× 0.80) slightly towards the end of ripening. However, the enzyme was most active in the ripe fruit, when measured at pH 7 [16].

Separation of acidic and alkaline FDPases

The two FDPases were separated on fractionation with ammonium sulfate (Table 1). Attempts to separate the activities employing calcium phosphate gel adsorption as described earlier in the case of banana FDPase [14] were unsuccessful. The acidic FDPase activity was precipitated in the 2nd ammonium sulfate fraction with *ca* 44% recovery of total activity whereas the alkaline enzyme was recovered in the 3rd and 4th ammonium sulfate fractions. The sp act of the alkaline component was higher (× 2) in the 2nd ammonium sulfate fraction than in the 4th fraction. The alkaline FDPase was purified *ca* 4-fold and the acidic FDPase was purified *ca* 3-fold (Table 1).

The activity of both preparations was tested in pH ranges 3–10. The pH optimum was 6 in the case of acidic FDPase and 8.5 in the case of alkaline FDPase, confirming the presence of two well separable FDPase activities in mango.

Substrate specificity

The two activities separated from mango were specific for FDP. They did not hydrolyse G-6-P, G-1-P, F-6-P, F-1-P, β-glycerophosphate or pyrophosphate under the assay conditions described. The two FDPases differed in their response towards the substrate FDP, the alkaline form showing maximum activity at 0.3 mM FDP and the acidic form at 0.2 mM FDP. The affinity towards FDP was greater with alkaline FDPase ($K_m = 0.154 \times 10^{-3}$ M) than with acidic FDPase ($K_m = 1.67 \times 10^{-3}$ M). However the acidic form showed a higher rate of reaction (V_{max} = 25 μmol Pi/mg protein/hr) as compared to the alkaline form (V_{max} = 10 μmol Pi/mg protein/hr).

Table 1. Purification of FDPase from mango

Fraction	Volume (ml)	Protein mg/ml	*Specific activity		Total activity (units)	
			Acidic	Alkaline	Acidic	Alkaline
Crude homogenate	100	0.85	4.2	1.9	360	162
Magnesium acetate-supernatant	98	0.82	4.3	2.0	346	162
Ammonium sulfate fractionation:						
1st fraction						
0–10% saturation	25	0.54	6.8	4.9	92	66
2nd fraction						
10–30% saturation	10	0.92	0	7.8	0	72
3rd fraction						
30–60% saturation	10	1.01	14.5	0	145	0
4th fraction						
60–90% saturation	10	1.25	7.3	0	92	0

* sp. act refers to units of enzyme activity per mg protein.

Metal requirements

Omission of either Mg^{2+} or Mn^{2+} ions from the reaction mixture resulted in loss of enzyme activity. Both FDPases showed similar responses towards the divalent metal ions. Mg^{2+} gave less activation than Mn^{2+} ($\times$ 0.5), and Ba^{2+} was intermediate. Maximum response was observed at 15 μmol concn of the metal ions in the reaction mixture (2 ml). A reduction in FDPase activity was noted when either Zn^{2+} or Co^{2+} was included in the assay system. Both forms of mango FDPases are thus different from banana FDPases, for which Mg^{2+} ions were more effective than Mn^{2+} ions in activating the reaction [14].

Effect of AMP and EDTA

FDPase is an allosteric enzyme and AMP functions as a negative allosteric effector in regulating the enzyme activity *in vivo*. Treatment of the enzyme with EDTA also brought about an additional peak of activity in the case of *Dictyostelium discoidium* FDPase [19]. In order to compare the catalytic nature of acidic and alkaline FDPases from mango, the effect of these two reagents was tested. The acidic FDPase of mango showed a lesser degree of inhibition with increasing concentration of AMP, whereas the inhibition observed with respect to the alkaline form was greater. An inhibition of activity as high as 93% was observed in the case of alkaline FDPase as compared to a value of 68% in the case of acidic FDPase, when the AMP concentration was 10^{-4} M in the reaction mixture. A similar observation has been made by other workers [14] that the two FDPase activities observed in irradiated banana responded differentially to AMP *in vitro*.

Preincubation of alkaline FDPase with EDTA before the addition of the substrate FDP, resulted in a greater activation (75%), whereas only a 10% activation was observed in the case of acidic FDPase.

Temperature stability

The temperature stabilities of the two FDPases have been compared at 50°. The acidic FDPase showed a sharp fall in activity (48% residual activity) within 20 min of holding at 50°, whereas the alkaline component showed *ca* 90% retention in activity during the same period.

EXPERIMENTAL

Alfonso mangoes (*Mangifera indica*) used for this study were obtained from the local market. FDPase assay with the crude extract was carried out at pH 6 (Tris–maleate buffer), according to the procedure of ref. [15], omitting cysteine and $MnCl_2$ from the reaction mixture. The reaction mixture consisted of 5 μmol $MgCl_2$ and appropriate amount of enzyme (100–125 μg protein for the crude enzyme and 25 μg for the purified preparation) in a total vol of 2 ml. The reaction mixture was incubated for 1 hr at 37°. The product of the reaction was determined by estimating Pi [17] liberated after stopping the reaction with 10% TCA. A unit of enzyme activity was defined as the amount of enzyme which liberates 1 μmol of Pi under standard assay conditions. Sp act refers to units per mg protein. Protein determinations were carried out according to the method of ref. [18].

Purification of FDPase. Fresh preclimacteric mangoes were frozen at −20° after the removal of peel and stones and ground with 5% PVP in a precooled mortar. A homogeneous slurry was then made using an equal vol of 0.1 M Tris buffer, pH 7.5 and it was centrifuged at 10000 *g* for 30 min. The supernatant was used as the crude enzyme preparation. The extraction procedure was conducted between 0 and 5°. To the crude extract, solid Mg $(OAc)_2$ was added to a final concn of 10 mM. The extract was cooled to 0–5° for 30 min and centrifuged at 10000 *g* for 15 min. To the supernatant, solid $(NH_4)_2SO_4$ was added to give a final concn of 10%. The soln was left for 2 hr and then centrifuged at 15000 *g* for 15 min. The ppt (1st fraction) was collected and dissolved in 25 ml of 0.1 M Tris buffer, pH 7.5 (see Table 1). The supernatant was treated in a similar way each time the salt concn was raised, the ppt was collected and dissolved in buffer. The 4 fractions were then dialysed against 50 mM Tris buffer, pH 7.5 for 2 hr.

Effect of fructose 1,6-diphosphate concn on the activity of FDPases. Experimental details are the same as described above except different concns of F-1,6-diphosphate (0.1–0.5 μmol) were included in the reaction mixture.

Effect of AMP and EDTA. The enzyme was preincubated for 5 min with different concns of AMP (10^{-6}–10^{-4} M) before the addition of the substrate and subsequent determination of enzyme activity. A control without AMP was also set up. To study the effect of EDTA on the enzyme activity, preincubation of the enzyme with 0.5 mM EDTA for 5 min was carried out followed by assay of the enzyme activity.

Thermal stability. The two purified FDPases were held at 50° and residual activities were measured at various time intervals up to 100 min.

Acknowledgements—This investigation was partly supported by a PL-480/FG-In-506 grant from the Agricultural Research Service of the U.S. Department of Agriculture. One of the authors (N.N.R), kindly acknowledges C.S.I.R. (India) for

the grant of a senior research fellowship during the tenure of this work.

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