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PHOSPHOFRUCTOKINASE FROM IMMATURE WHEAT GRAINS

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Key Word Index—*Triticum aestivum*, Gramineae, wheat, grain, phosphofructokinase, glycolysis, regulation

Abstract—Levels of phosphofructokinase and metabolites known to affect its activity were monitored at different stages of wheat grain development Phosphofructokinase activity peaked at 28 days after anthesis, declining thereafter The amount of citrate increased up to 14 days after anthesis PEP, ATP, ADP and AMP showed peak values at 28 days after anthesis Phosphofructokinase from 28-day-old grains was purified × 23 with 49% recovery by ammonium sulphate fractionation and chromatography on DEAE-Sephadex A-50 A normal hyperbolic curve was observed with F-6-P ATP inhibited the enzyme above 0 75 mM ADP, citrate and 2-P-glycolate inhibited the enzyme non-cooperatively, K_i values being 2 2, 1 6 and 5 0 mM, respectively PEP and AMP failed to inhibit the enzyme activity

INTRODUCTION

Development in cereal grains including wheat, is characterized by a net synthesis and deposition of reserve materials, mainly starch [1] A constant energy supply is to be maintained to ensure these synthetic processes High respiratory activities during grain development in barley [2] and wheat [3], and grouping of mitochondria around amyloplasts [4] also show that synthesis of reserve materials during grain development needs constant energy supply Glycolysis is an important pathway of the energy yielding processes *In vitro* studies with purified phosphofructokinase preparations from carrot [5], pea seeds [6], potato tubers [7] and spinach leaf chloroplast [8], and intact tissue studies [9, 10] have shown that glycolysis is controlled at the phosphofructokinase step If this enzyme exerts such a control in developing cereal grains also, it may have an important bearing on the rate of energy dependent starch synthesis However, kinetic details of this enzyme from cereal grains are not available The present investigation was aimed at characterizing this enzyme from immature wheat grains to determine its possible regulatory role

RESULTS AND DISCUSSION

Phosphofructokinase

The activity of phosphofructokinase increased steadily from 7 DAA to reach a maximum at 28 day stage, declining thereafter (Fig 1) Duffus and Rosie [2] in barley and Sangwan *et al* [3] in wheat have also observed peak values for this enzyme at 28 DAA

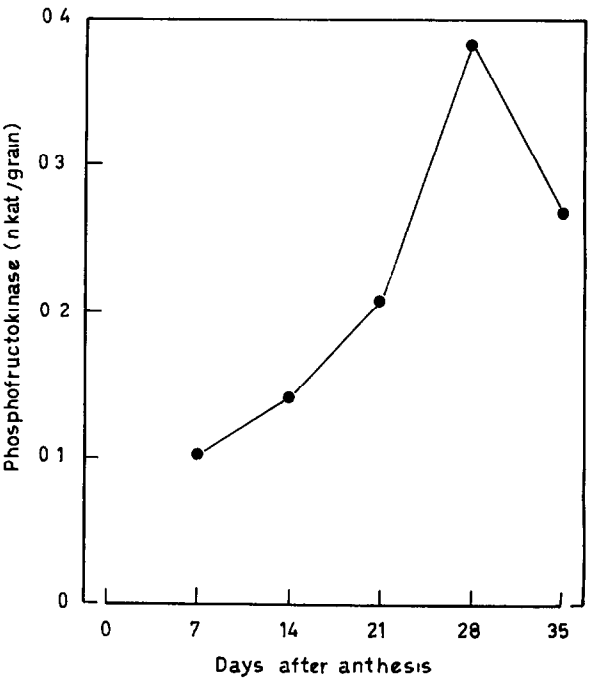

Fig 1 Activity of phosphofructokinase during wheat grain development

Metabolites

The levels of those metabolites which are known to affect PFK activity, such as PEP, citrate, AMP, ADP and ATP, were monitored at different developmental stages Peak levels (nmol/grain) of citrate and PEP were observed at 14 and 28 DAA, respectively (Fig 2), whilst peak levels for adenylate nucleotides were observed at 28 DAA (Fig 3)

Partial purification and characterization

The enzyme phosphofructokinase was purified × 23 with *ca* 49% recovery using ammonium sulphate fractionation and DEAE-Sephadex A-50 column chromatography (Table 1) The pH optimum of the enzyme was 7 8 and optimum temperature 35° The enzyme was quite stable, as it could retain 85% of the original activity after storage at 4° for 10 days

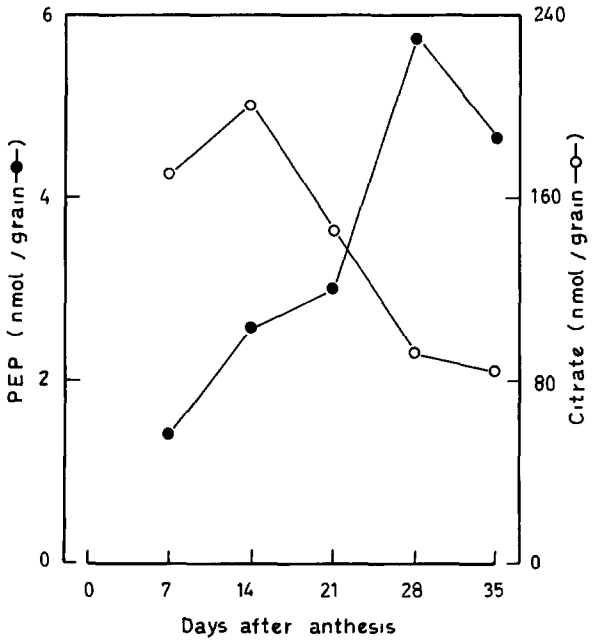

Fig 2 Citrate and PEP level during wheat grain development

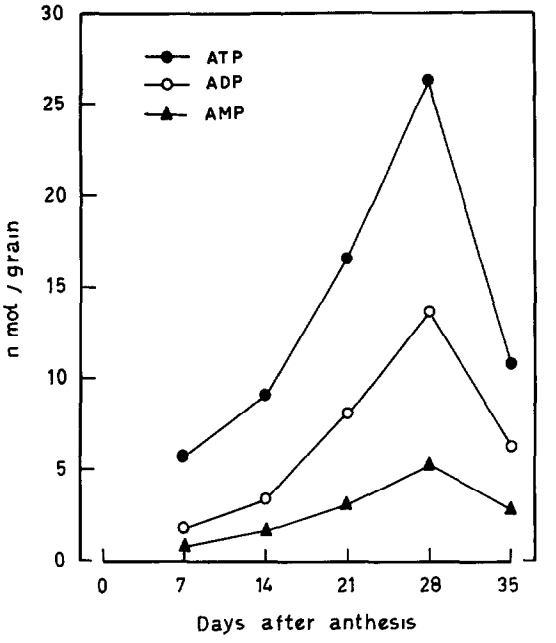

Fig 3 ATP, ADP and AMP level during wheat grain development

Effect of substrates

The response of phosphofructokinase activity to F-6-P was studied up to 20 mM, keeping ATP and Mg^{2+} at 0 75 and 5 mM, respectively A sharp increase in the enzyme activity was observed up to 5 mM, above which the activity remained almost constant indicating that the enzyme followed Michaelis–Menten kinetics (Fig 4) From a double reciprocal plot, the K_m for F-6-P was 1 2 mM

While studying the effect of ATP, the concentrations of both F-6-P and Mg^{2+} were kept constant at 5 mM Maximum activity was observed at 0 75 mM ATP (Fig 5) Above this ATP had a strong inhibitory effect, which was more pronounced up to 1 5 mM Free ATP inhibition has also been reported for carrot [5, 11] and spinach leaf chloroplast [8] phosphofructokinase preparations However, in the present case, ATP inhibition was observed even when the Mg^{2+} concentration was far in excess of that of ATP, indicating that even the $MgATP^{2-}$ complex inhibits the enzyme activity This property of the wheat grain enzyme resembles that of the castor bean endosperm [12] and the green alga *Dunaliella marina* [13]

Unless otherwise stated, further experiments were carried out at Mg^{2+}, F-6-P and ATP concentrations of 5 0, 5 0 and 0 75 mM, respectively

Effect of metal ions

The enzyme preparation showed an absolute requirement for Mg^{2+}, for which 5 mM was saturating No activity was observed with any other ion tested (Na^+, K^+, NH_4^+, Co^{2+}, Sr^{2+}, Ca^{2+} and Mn^{2+}) Inclusion of these ions up to 10 mM with Mg^{2+} also had no effect on the enzyme activity, except Sr^{2+} which had a slight inhibitory effect (20% at 10 mM)

Effect of metabolites

Studies conducted with purified phosphofructokinase preparations from various tissues [5–8, 13–18] have shown that it is subjected to regulation mainly by ADP, citrate, PEP, AMP and 2-P-glycolate Hence the effect of these metabolites was studied From double reciprocal plots, ADP was found to be a non-competitive inhibitor, whereas citrate and 2-P-glycolate inhibited the enzyme

Table 1 Purification of phosphofructokinase from immature wheat grains

Fraction	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg protein)	Purification (fold)	Recovery (%)
Crude extract	1440	85 0	0 059	—	100
$(NH_4)_2SO_4$(25–45%)	305	69 4	0 227	3 85	81 7
DEAE-Sephadex A-50	24	33 3	1 37	23 2	48 9

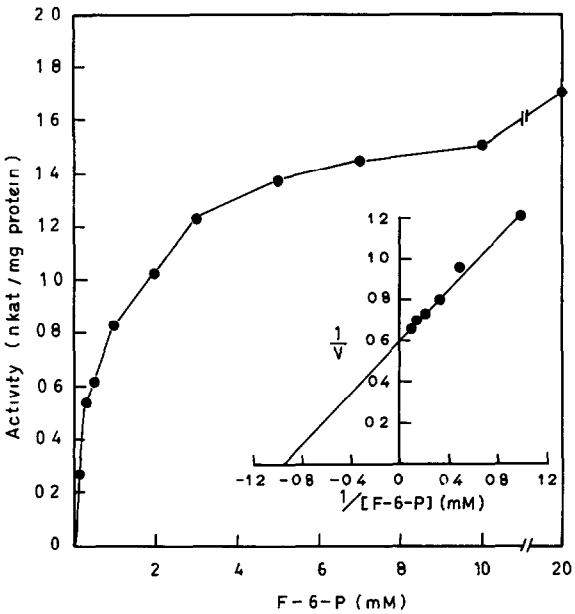

Fig 4 Effect of F-6-P on the activity of phosphofructokinase (inset—double reciprocal plot)

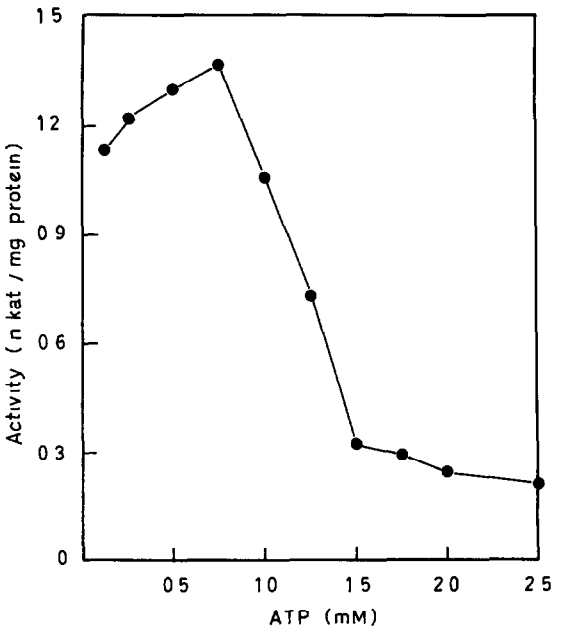

Fig 5 Effect of ATP on the activity of phosphofructokinase

competitively K_i values for ADP, citrate and 2-P-glycolate as determined from Dixon's plot were 2 2, 1 6 and 5 0 mM, respectively Hill coefficient values of 0 96 (ADP), 0 95 (citrate) and 1 08 (2-P-glycolate) as calculated from Hill plots ruled out the possibility of cooperative interaction of these metabolites AMP and PEP failed to inhibit the enzyme up to as high as 6 mM concentrations, showing that phosphofructokinase from wheat grains is far less sensitive to inhibition by these metabolites as compared to the enzyme from other sources This enzyme has earlier also been shown to be less sensitive to various inhibitors from different sources [15, 19–21] In this regard, the corollary can be drawn from ADPG-pyrophosphorylase, an important enzyme of starch biosynthesis This enzyme from leaves of different sources was activated and inhibited up to 50-fold at very low levels of 3-PGA and Pi [22, 23] However, from non-photosynthetic storage tissues like maize endosperm and wheat grain, it was found to be 100–150 fold less sensitive [24, 25]

The concentrations of ATP, ADP and citrate required to cause significant inhibition of the enzyme *in vitro* were much higher than their physiological concentrations Only citrate, when maximum at the concentration approached this *in vitro* inhibitory level However, this concentration was attained only at 7 DAA when starch deposition was negligible Thus unless very high concentrations of these metabolites are assumed to be present at the site of phosphofructokinase, they cannot be implicated in the process of regulation of phosphofructokinase This together with the absence of cooperative interaction by these metabolites suggest that glycolysis in wheat grains may not be regulated at the step of phosphofructokinase In corn scutellum also, glycolysis was found not to be regulated at the phosphofructokinase step [26]

EXPERIMENTAL

Plant material Wheat crop (cv WH-157) was raised under field conditions following recommended agronomic practices Ear heads were harvested and processed as described in ref [27]

Enzyme extraction and assay Whole grains in duplicate were hand homogenized in 5 mM imidazole–HCl buffer (pH 7 7) containing 1 mM EDTA, 4 mM $MgCl_2$ and 5 mM β-mercaptoethanol Out of several media containing tris, triethanolamine, glycylglycine and imidazole, this medium gave maximum extraction of native phosphofructokinase The homogenate was centrifuged at 15 000 *g* for 30 min at 0–2° The pellet was washed (× 2) with extraction buffer and recentrifuged The combined supernatant made to a known vol after dialysis against the extraction medium served as the enzyme preparation The pellet was free of enzyme activity Phosphofructokinase was assayed by following the procedure of ref [28] Preliminary experiments were carried out to optimize assay conditions with respect to pH, temp and buffer The reaction could be started by both F-6-P and ATP Linear reaction rates were obtained up to 20 min Blank values due to auto-oxidation of NADH (0 002 per 3 min) were subtracted

Metabolite extraction and assay Metabolites were extracted according to ref [29] Estimation of PEP was done following the formation of pyruvate by pyruvate kinase and lactate dehydrogenase coupling [30] Citrate was estimated by the procedure of ref [31] The method of ref [32] was followed for ATP estimation ADP and AMP were estimated according to ref [33] The extraction and estimation of all the metabolites was completed within 6 hr of grain harvest The representative quantities of biochemicals (0 5 μmol) added exogenously before homogenization with $HClO_4$ could be quantitatively recovered and measured, recoveries being in the range of 82–90%

Enzyme purification All purification steps were carried out at 0–4° Whole grains (40 g) from earheads harvested at 28 DAA were hand homogenized in extraction buffer described above The extract was squeezed through two layers of cheese cloth and centrifuged at 15 000 *g* for 30 min The fraction obtained between

25–45% $(NH_4)_2SO_4$ saturation was layered on DEAE-Sephadex A-50 column (30 × 2 5 cm) previously equilibrated with extraction buffer A gradient was established with NaCl (0 1–0 4 M) The enzyme was eluted at 0 2 M NaCl The active fractions were pooled and the preparation thus obtained was used for all experiments described here The enzyme preparation was free of FBPase and β-NADH oxidase activities Each experiment was performed in triplicate Protein was determined according to ref [34]

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