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ALKALINE INORGANIC PYROPHOSPHATASE FROM IMMATURE WHEAT GRAINS

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; alkaline inorganic pyrophosphatase; partial purification; regulation; starch biosynthesis; intermediates.

Abstract—Changes in the level of alkaline inorganic pyrophosphatase and ADPG-pyrophosphorylase were monitored in developing wheat grains at weekly intervals after anthesis. The enzyme activities increased to reach a maximum at 28 days after anthesis, whereupon they declined. Alkaline inorganic pyrophosphatase from 25- day-old grains was purified 107-fold. It acted optimally at: 35°, Mg^{2+}: PPi :: 1:2, pH 7.3, with a K_m value of 2.42 mM. The preparation exhibited high specificity for PPi. Mg^{2+} was the most effective cofactor. F^- and NO_3^- inhibited the enzyme. Iodoacetamide did not cause any inhibition. None of the intermediate metabolites of sucrose–starch conversion had any effect on the activity of the enzyme.

INTRODUCTION

Starch biosynthesis in developing cereal grains is brought about by starch synthetases which utilize both ADPG and UDPG as substrates. However, ADPG is more active as the glucosyl donor than UDPG [1, 2]. ADPG in the developing grain is produced by the action of ADPG-pyrophosphorylase, which is thought to be a regulatory enzyme in starch biosynthesis [3]. Since PPi, a product of the ADPG-pyrophosphorylase reaction, inhibited sucrose–starch conversion in sweet corn [4], its accumulation in developing cereal grain might regulate the equilibrium of the reaction catalysed by the above enzyme. As the level of PPi in developing grain is controlled by the enzyme alkaline inorganic pyrophosphatase (EC 3.6.1.1) which hydrolyses PPi to Pi and does not allow it to accumulate, it may very well be a vulnerable point in the control of starch biosynthesis. Inhibition of ADPG-pyrophosphorylase by PPi [5] further strengthens this belief. Though the presence of pyrophosphatase has already been indicated in a number of developing cereal grains [6–8], its characteristics have yet to be investigated. Thus, it seemed of interest to investigate the properties and behaviour of this enzyme during wheat grain development with a view to gain some information on its role in the control of starch biosynthesis.

RESULTS AND DISCUSSION

Developmental pattern

The activity of alkaline inorganic pyrophosphatase was low during the earlier stages of grain development but increased steadily from 7 days after anthesis to reach a maximum at 28 days and then declined until maturity (Fig. 1). The pattern was similar to that of ADPG-pyrophosphorylase, indicating the possibility that it ensures the removal of PPi released in the reaction catalysed by ADPG-pyrophosphorylase.

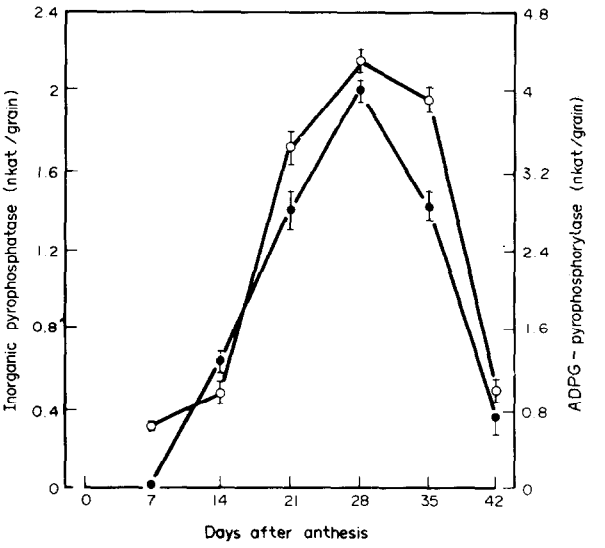

Fig. 1. Activity of alkaline inorganic pyrophosphatase (○——○) and ADPG-pyrophosphorylase (●——●) during wheat grain development.

Purification and characterization

The enzyme was purified *ca* 107-fold with 40% recovery calculated on the basis of the crude extract (Table 1). At the optimum temperature (35°), the velocity of the reaction was linear with respect to enzyme concentration over a 5-fold range (10–50 μg protein). All the experiments to be described were conducted under conditions where linearity with respect to time and enzyme concentration was observed.

Optimum pH

While investigating the effect of pH over the range of

Table 1. Purification of alkaline inorganic pyrophosphatase from immature wheat grains

Fraction	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg protein)	Purification (fold)	Recovery (%)
Crude extract	319.0	158.9	0.49	—	100
$(NH_4)_2SO_4$ (40–70%)	52.3	103.3	1.97	4.0	65.0
Sephadex G-200	2.2	78.0	35.45	72.4	49.1
DEAE-cellulose	1.2	63.3	52.78	107.7	39.8

4.0–9.0, the ratio of substrate (2.5 mM) to Mg^{2+} (1.25 mM) was kept at 2:1. The buffers used in different pH ranges were: 0.05 M acetate (pH 4.0–5.5), 0.05 M Tris–maleate (pH 5.5–7.0) and 0.05 M Tris–HCl (pH 7.0–9.0). Irrespective of the presence or absence of Mg^{2+}, the enzyme exhibited two pH optima, 5.0 and 7.3 (Fig. 2). The activity in the absence of Mg^{2+} was, however, less; the effect being more pronounced in the alkaline range. However, the enzyme was not active in the absence of Mg^{2+} above pH 8.2. The pH optimum in the alkaline range is lower than those reported for this enzyme from sugarcane [9], corn leaves [10, 11], corn endosperm [7] and guar cotyledons [12]. It also differs from the preparations of Maslowska and Maslowski [7] and Popli and Singh [12] in the sense that activity in the absence of Mg^{2+} was detectable in the alkaline range. Though the activity chromatographed as a single peak both on Sephadex and DEAE-cellulose, the two pH optima indicate that either the purification was not sufficient to exclude acid inorganic pyrophosphatase, or the same enzyme exhibited activity in both ranges.

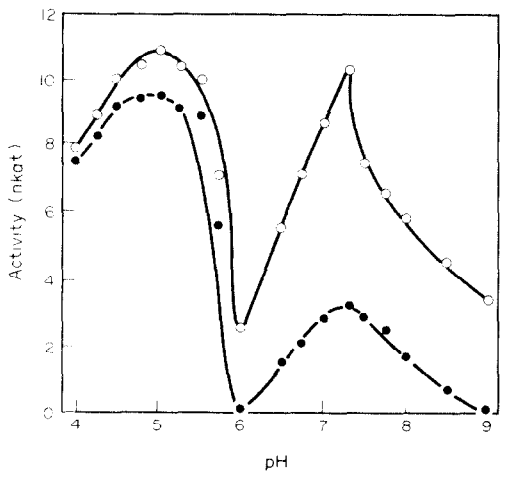

Fig. 2. Alkaline inorganic pyrophosphatase activity at different pH values in the presence (○——○) and absence (●——●) of Mg^{2+}.

Magnesium and pyrophosphate ratio (Mg^{2+}: *PPi*)

With substrate concentrations of 2.5 and 5 mM, the enzyme showed maximum activity when the Mg^{2+}: PPi ratio was 1:2. At higher concentrations of Mg^{2+}, the Mg^{2+}: PPi ratios for optimal activity were 1:1, 2:1 and 5:1, the activities observed being some 92, 49 and 44%, respectively of those obtained with a ratio of 1:2. The optimum ratio observed here is less than those reported earlier [7, 9, 11, 12].

At the pH of our assay, essentially all the pyrophosphate was present as $P_2O_7^{4-}$, thus giving rise to both monomagnesium pyrophosphate ($MgP_2O_7^{2-}$) and dimagnesium pyrophosphate ($Mg_2P_2O_7$) on combination with Mg^{2+} [13]. Since maximum activity was observed at low Mg^{2+} concentration, $MgP_2O_7^{2-}$ appears to be the preferred substrate.

Effect of Mg^{2+} *concentration on* pH *optima*

On keeping the substrate concentration constant at 2.5 mM, the activity of the enzyme increased as the Mg^{2+}: PPi ratio was lowered from 5:1 to 1:2, up to pH 7.8. However, at higher pH values (8.0–9.0), the reverse trend was observed. There was a shift in pH optimum from 7.3 to 8.2 at high Mg^{2+}: PPi ratio (5:1). However, the activity at pH 8.2, and with a Mg^{2+}: PPi ratio of 5:1, was only 55% of that observed at pH 7.3 with a Mg^{2+}: PPi ratio of 1:2. This indicates that pH values above 8.0 facilitate the formation of $Mg_2P_2O_7$, which is a poor substrate compared to $MgP_2O_7^{2-}$, the formation of which is favoured at lower pH values. Other workers had earlier reported that optimum pH value is a function of Mg^{2+} and PPi concentrations [10, 12, 14].

Effect of substrate concentration

The enzyme activity increased with increasing substrate concentration to reach a maximum at 10 mM above which the activity remained almost constant, indicating that the enzyme activity followed Michaelis–Menten kinetics. From a double-reciprocal plot, the K_m for the substrate was found to be 2.42 mM, which compares well with that of maize [10] and guar enzymes [12], but is higher than that of sugarcane enzyme, for which a value of 0.75 mM was obtained [9].

Substrate specificity

The enzyme was highly specific for PPi. It caused no hydrolysis of phosphorylated compounds such as glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1-6-diphosphate, ATP, ADP, AMP and $NADP^+$. These findings showed that the enzyme preparation was free of any phosphomonoesterase or phosphodiesterase activity.

Effect of anions and cations

The effect of chloride salts of various divalent cations was studied at a concentration of 2.5 mM. Mg^{2+} was the

most efficient activator. Among the other cations tested, Mn^{2+}, Zn^{2+}, Co^{2+} and Ca^{2+} activated the enzyme only by 36, 23, 20 and 7% respectively of that caused by Mg^{2+}.

The effect of different anions on enzyme activity was studied by using the appropriate magnesium salts. Only F^- and NO_3^- had strong inhibitory effects (75%). SO_4^{2-} partially inhibited the enzyme (16%), whilst Cl^-, Br^- and I^- failed to inhibit the enzyme at the concentration used (2.5 mM).

Effect of thiol reagents

Preincubation of enzyme with cysteine, glutathione and β-mercaptoethanol at concentrations up to 5 mM failed to activate the enzyme, indicating that it does not require thiol groups for its activity. This observation confirms those reported earlier [10, 12].

Effect of metabolites

The effect of intermediate metabolites of sucrose–starch conversion and a few other metabolites was studied on the activity of this enzyme to ascertain whether or not it can be implicated in the regulation of starch biosynthesis in developing wheat grains. At concentrations of 1.25, 2.5 and 5 mM, sucrose, fructose-6-phosphate, glucose-6-phosphate, glucose-1-phosphate, ADPG, GDPG, UDPG, AMP, ADP, ATP, GDP, UDP, UTP, NAD^+, $NADP^+$, NADPH and fructose-1-6-diphosphate were without effect on the activity of this enzyme, suggesting that this enzyme may not be involved in the regulation of starch biosynthesis during grain development in wheat. However, the low activity of this enzyme up to 14 days after anthesis and its high activity during the active phase of starch accumulation may indicate that at early stages the equilibrium will favour glucose-1-phosphate formation from UDPG and at later stages, the increase in pyrophosphatase activity would allow synthesis of ADPG by a reversal of equilibrium. But whether the PPi inhibition is selective in the cases of UDPG- and ADPG-pyrophosphorylases is yet to be determined. However, if the inhibition is selective, then, as suggested by Duffus [15], a coupling of UDPG-pyrophosphorylase in the direction of glucose-1-phosphate synthesis with ADPG-pyrophosphorylase in the direction of ADPG-synthesis would ensure a rapid conversion of sucrose to starch. This aspect needs further investigation.

EXPERIMENTAL

Plant material. Wheat crop (cv WH-157) was raised under field conditions following recommended agronomic practices. Earheads harvested first at 7 days after anthesis and then at weekly intervals until maturity (42 days after anthesis) were processed further as described in ref. [16].

Enzyme extraction. Whole grains in duplicates were hand-homogenized in 0.05 M Tris–HCl buffer (pH 7.0). The homogenate was centrifuged at 10000 *g* for 30 min at 0–2°. The pellet was washed (× 2) with extraction buffer and recentrifuged. The combined supernatants made up to a known vol. served as the enzyme preparation. The pellet was shown to be free of any inorganic pyrophosphatase and ADPG-pyrophosphorylase activity.

Enzyme assay. The two extracts were assayed in duplicate for both enzymes. The assay mixture (4 ml) for inorganic pyrophosphatase contained 2.5 mM Na pyrophosphate, 1.25 mM $MgCl_2$, 0.05 M Tris–HCl buffer (pH 7.3) and 0.5 ml enzyme extract. Following incubation at 37° for 15 min, 1 ml of 20% TCA was added. The precipitated protein was removed by centrifugation and liberated Pi determined by the method described in ref. [17]. In the control, TCA was added before enzyme addition. ADPG-pyrophosphorylase was assayed by coupling the product of reaction with phosphoglucomutase and glucose-6-phosphate dehydrogenase [16]. The reaction was started by addition of PPi.

Enzyme purification. All purification steps were carried out at 0–4°. Whole grains (50 g) from ears harvested at 25 days after anthesis were homogenized in 0.05 M Tris–HCl buffer (pH 7.3). The extract was squeezed through cheesecloth and spun down at 16000 *g* for 30 min. The supernatant was then subjected to $(NH_4)_2SO_4$ fractionation. The fraction obtained between 40 and 70% saturation was subjected to Sephadex G-200 chromatography (60 × 3 cm, flow rate 8 ml/hr), previously equilibrated with 0.05 M Tris–HCl buffer (pH 7.3), and eluted with the same buffer. The active fractions (35–50 of 4 ml each) were pooled and the protein precipitated by $(NH_4)_2SO_4$. The ppts. were dissolved in Tris–HCl buffer (pH 7.3) and dialysed free of ions. The dialysate was passed through a DEAE-cellulose column (30 × 2 cm). A gradient was established with NaCl (0.2–0.6 M). The enzyme was eluted at 0.3–0.4 M NaCl. The active fractions were pooled and the enzyme preparation thus obtained was used for all experiments described here. Each experiment was performed in triplicate. Protein was determined according to ref. [18].

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REFERENCES

1. Murata, T., Sugiyama, T. and Akazawa, T. (1964) *Arch. Biochem. Biophys.* **107**, 92.
2. Recondo, E. and Leloir, L. F. (1961) *Biochem. Biophys. Res. Commun.* **6**, 85.
3. Preiss, J. and Levi, C. (1979) in *Encyclopedia of Plant Physiology* (Gibbs, M. and Latzko, E., eds.), Vol. 6, p. 282. Springer, Berlin.
4. Amir, J. and Cherry, J. H. (1971) *J. Agric. Food. Chem.* **19**, 954.
5. Amir, J. and Cherry, J. H. (1972) *Plant Physiol.* **49**, 893.
6. Baxter, E. D. and Duffus, C. M. (1973) *Phytochemistry* **12**, 1923.
7. Maslowska, H. and Maslowski, P. (1975) *Z. Pflanzenphysiol.* **76**, 307.
8. Batra, V. I. P. and Mehta, S. L. (1981) *Phytochemistry* **20**, 1827.
9. Bucke, G. (1970) *Phytochemistry* **9**, 1303.
10. Simmons, S. and Butler, G. L. (1969) *Biochim. Biophys. Acta* **172**, 150.
11. Rip, J. W. and Rauser, W. E. (1971) *Phytochemistry* **10**, 2615.
12. Popli, S. and Singh, R. (1977) *Phytochemistry* **16**, 885.
13. Lambert, S. M. and Walters, J. I. (1957) *J. Am. Chem. Soc.* **79**, 5606.
14. El-Badry, A. M. and Bassham, J. A. (1970) *Biochim. Biophys. Acta* **197**, 308.
15. Duffus, C. M. (1979) in *Recent Advances in the Biochemistry of Cereals* (Laidman, D. L. and Wynjones, R. G., eds.), p. 209. Academic Press, New York.
16. Kumar, R. and Singh, R. (1980) *Phytochemistry* **19**, 2299.
17. Ozbun, J. L., Hawker, J. S., Greenberg, E., Lammel, C. and Preiss, J. (1973) *Plant Physiol.* **51**, 1.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.