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PROPERTIES OF ACID PHOSPHATASE FROM SCUTELLA OF GERMINATING MAIZE SEEDS

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Abstract—One acid phosphatase (optimum pH at 5.4) was purified from maize scutellum after 96 hr of germination. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis (PAGE) with or without sodium dodecyl sulfate (SDS). The enzyme has a MW of 65000 ± 4000 as determined by Sephadex G-200 gel filtration and SDS-PAGE. The enzyme contained 16% neutral sugars, and cations are not required for activity. The purified enzyme was not inactivated by DTNB at pH 8. The hydrolysis of glucose-6-phosphate in the presence of 4 mM fluoride and 4 mm EDTA, at pH 6.7 (optimum pH), seems to be catalysed by this acid phosphatase.

INTRODUCTION

It has been suggested that higher plants contain both a non-specific acid phosphatase and a specific glucose-6-phosphatase [1–3]. The evidence for the latter claim is not entirely convincing, as the possibility that the activity with G-6-P was due to some other phosphomonoesterase was not excluded [4]. Moreover, the presence of a specific glucose-6-phosphatase in higher plants may be of uncertain metabolic significance [2].

In some instances, however, the observed hydrolysis of G-6-P at appropriate pH (6.5) and substrate concentration was considered sufficient evidence to demonstrate the presence of a specific glucose-6-phosphatase [5]. One approach used to minimize this problem is to assay G-6-P hydrolysis in the presence of 4 mM KF and 4 mM EDTA, which were reported [6] to inhibit almost completely intestinal acid and alkaline phosphatase but not glucose-6-phosphatase. The other is to assay G-6-P hydrolysis with homogeneous preparations of the enzyme.

This paper describes the purification and some properties of the acid phosphatase, present in germinated maize cotyledons, which also catalyses the hydrolysis of G-6-P. Our results suggest that the observed hydrolysis of G-6-P in the presence of 4 mM KF and 4 mM EDTA, at pH 6.7 (the optimum pH), was catalysed by this enzyme.

RESULTS AND DISCUSSION

It has been shown that many seeds contain an acid phosphatase activity which increases considerably during germination and then slowly disappears [7]. The maize seeds described here showed a similar pattern with a 60-fold increase in activity after 96 hr of germination. 75–80% of the total extractable acid phosphatase activity of the whole seeds (see Experimental) is present in scutella, and after centrifugation for 2 hr at 105000 *g*, 95% of this activity was recovered in the soluble fraction. Glucose-6-phosphatase activity also increased during germination (Fig. 1), and was also found in the 105000 *g* supernatant. Similar results were reported for castor bean endosperm, in which glucose-6-phosphatase activity does not appear to be associated with any particulate fraction [5]. Contrary to that reported for the glucose-6-phosphatase activity from *Phaseolus vulgaris* cotyledons [8], the *Zea mays* enzyme was not inhibited by the 20000 *g* or 105000 *g* sediments of the extracts. Also, no dialysable inhibitors or activators were found in the crude extract of scutella for both activities.

In an attempt to recognize whether these two phosphatase activities were due to a single protein, a purification procedure, summarized in Table 1, was carried out. At the final step, the enzyme was purified 122-fold over the crude extract with an overall yield of 16%.

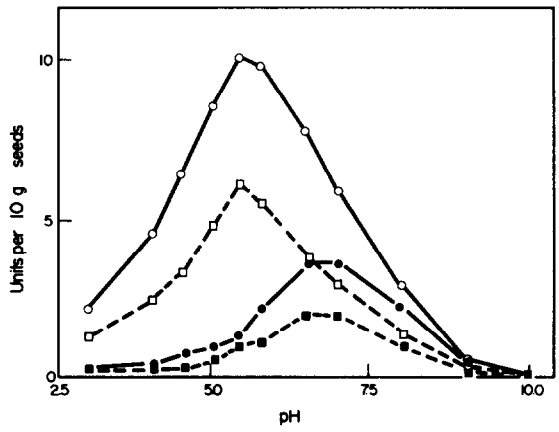

Fig. 1. pH Activity profile of the 105000 *g* supernatant on G-6-P. □, ■ represent acid phosphatase and glucose-6-phosphatase activities, respectively, at 48 hr of germination. ○, ● represent acid phosphatase and glucose-6-phosphatase activities at 96 hr of germination (see Experimental).

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Table 1. Purification of acid phosphatase from maize scutellum after 96 hr of germination

Fraction	Volume (ml)	Total activity (units) (a)	(b)	Specific activity (units/mg) (a)	(b)
I. Crude extract	425	468	130	0.3	0.08
II. $(NH_4)_2SO_4$	23	329	88	4.0	1.1
III. DEAE-cellulose	46	299	74	7.4	1.8
IV. DEAE-Sephadex	6	173	48	19.2	5.3
V. Sephadex G-75	48	110	27	24.0	5.9
VI. 2nd DEAE-cellulose	8	76	20	36.5	9.7

(a) For extraction conditions and *p*-nitrophenylphosphatase activity (pH 5.4), see Experimental.

(b) Assays of G-6-P hydrolysis (pH 6.7) in the presence of 4 mM KF and 4 mM EDTA were performed under conditions described in Experimental.

All preparations appeared homogeneous on 7.5% PAGE at pH 8.3 (not shown).

The activity of acid phosphatase towards various substrates is shown in Table 2. β-Glycerylphosphate gave the highest activity and phenylphosphate, *p*-nitrophenylphosphate and G-6-P were also significantly hydrolysed. No activity toward bis(*p*-nitrophenyl)phosphate, was detected. The relative rate of hydrolysis of each of the four substrates remained essentially the same at each stage of the purification procedure.

The K_m values at pH 5.4 and 6.7 were 0.3×10^{-3} M and 1.6×10^{-3} M for *p*-nitrophenylphosphate, and 1.2×10^{-3} M and 6.6×10^{-3} M for a G-6-P, respectively.

The effect of several substances on *p*-nitrophenylphosphatase activity is shown in Table 3. Various divalent metal ions examined (except $HgCl_2$) showed little or no inhibitory effect on *p*-nitrophenylphosphatase activity, either at pH 5.4 or 6.7. Addition of 20 mM EDTA to the reaction mixture had no effect, suggesting that metals were not required for enzyme activity. $HgCl_2$, KH_2PO_4, $(NH_4)_6Mo_7O_{24}$ and Na_3BO_3 showed an inhibitory effect which was more marked at pH 6.7 that at pH 5.4. By contrast, fluoride had a stronger inhibitory effect at pH 5.4, i.e. ten times more fluoride was required at pH 6.7 to provide the equivalent extent of inhibition shown at pH 5.4 (Table 3). The enzyme activity was not affected by incubation with DTNB for 3 hr at 37°, suggesting that the enzyme is not SH-dependent.

The neutral sugar content of the purified enzyme was 16%, suggesting that it was a glycoprotein. The MW was 65 000 ± 4000, as determined by exclusion chromatography and by SDS-PAGE. Although these methods are not quite accurate for glycoproteins [9, 10], these results suggested that the enzyme was a monomer. According to these results, the enzyme isolated from maize scutella might be regarded as a high MW acid phosphatase (or a degradation product) having the general characteristics of these enzymes [11], except for the fact that it is tartrate-resistant (Table 3) and apparently soluble.

Determination of the pH activity profile in the absence or presence of 4 mM fluoride and 4 mM EDTA could be taken as evidence for the existence of an acid phosphatase and a glucose-6-phosphatase in the crude extract (Fig. 1). However, the same pH profile was obtained with the purified enzyme when β-glycerylphosphate, G-6-P or *p*-nitrophenylphosphate was assayed as substrates. Moreover, the effect of $HgCl_2$, KH_2PO_4 and $(NH_4)_6Mo_7O_{24}$ as well as K_m values for *p*-nitrophenylphosphate and glucose-6-phosphate also showed a dependence on pH values. Taken together, these results suggested that G-6-P hydrolysis could be due to a non-specific acid phosphatase. The apparent dislocation of optimum pH from 5.4 to 6.7 in the presence of fluoride could be due to ionization of the substrate [12] or to ionization of the enzyme leading to the interconversion between two forms resulting in different affinities for

Table 2. Substrate specificity, at pH 5.4 and pH 6.7, of purified maize scutellum acid phosphatase

Substrate	Final concentration (mM)	Relative activity* pH 5.4	pH 6.7
β-Glycerylphosphate	30	100	100
Phenylphosphate	6	83	85
p-Nitrophenylphosphate	6	79	78
Glucose-6-phosphate	30	57	54
Bis(*p*-nitrophenyl)phosphate	6	3	2

* Assays were performed (in the absence of fluoride and EDTA) under conditions described in the Experimental. Activities are expressed as % relative to that obtained with β-glycerylphosphate.

Table 3. Effect of various compounds on the *p*-nitrophenylphosphatase activity (at pH 5.4 and 6.7) of purified maize scutellum acid phosphatase

Addition	Final concentration (mM)	Relative activity* pH 5.4	pH 6.7
None	—	100	100
EDTA	20	98	98
$CaCl_2$	5	96	92
$MgCl_2$	5	90	99
$CuSO_4$	5	83	87
$HgCl_2$	0.005	88	12
$HgCl_2$	0.02	65	0
$ZnCl_2$	5	90	95
KH_2PO_4	5	54	19
$(NH_4)_6Mo_7O_{24}$	5	19	0
Na_3BO_3	5	80	49
KF	4	16	61
KF	50	0	10
Tartarate	10	97	98
Adenine	1	96	95
DTT	1	100	90
Glucose	5	95	96
Glucose	100	94	97

*Activities are expressed as % relative to that obtained without addition.

fluoride at the two pHs assayed [13]. Thus, under the experimental conditions employed in the present study, the observed hydrolysis of G-6-P at pH 6.7 in the presence of 4 mM fluoride was an insufficient criterion to establish the presence of a distinct glucose-6-phosphatase in scutella of maize seeds.

EXPERIMENTAL

The following reagents were purchased from the sources indicated: glucose-6-phosphate (monosodium salt), BSA, ovalbumin, trypsinogen, DEAE-cellulose and DTT were from Sigma; sodium glycerylphosphate, *p*-nitrophenylphosphate and DTNB were from Merck; Sephadex G-200; DEAE-Sephadex A-50, Sephadex G-75 and Blue Dextran were from Pharmacia.

Growth conditions. Seeds of maize (*Zea mays* cv 79–74, Divisão Regional Agrícola de Ribeirão Preto, SP) were surface-sterilized in 0.5% NaOCl and soaked for 24 hr in H_2O (1-day-old scutellum tissue). The seeds were then germinated in the dark at 30° over filter paper. The seeds were regularly watered with H_2O.

Tissue fractionation. Scutella were hand ground in ice-cold 50 mM $NaHCO_3$ buffer (pH 7.4) containing 0.3 M sucrose (2.5 ml buffer per g of seed), in a pestle and mortar without added abrasives. The crude homogenate was filtered through gauze and centrifuged at 20 000 *g* for 10 min. The supernatant fraction was centrifuged again at 105 000 *g* for 2 hr in a Spinco No. 40 rotor. The resulting supernatants were used as a source of enzyme. All steps were carried out at 4°. All pellets were resuspended in the buffer used for tissue extraction, recentrifuged and finally suspended in 2–5 ml of the same buffer.

Assay procedures. Enzymes assays were run in 0.10 M NaOAc buffer (pH 5.4) using 2 ml 6 mM *p*-nitrophenylphosphate or phenylphosphate as substrates at 37°. The reaction was stopped by the addition of 1 ml of 1 M NaOH, and *p*-nitrophenol or phenol absorbance was measured at 405 nm (E = 17 800/mol/cm) or 278 nm (E = 2600/mol/cm), respectively. Glucose-6-phosphatase activity was measured using the method of ref. [6] and by measuring the Pi liberated by the method of ref. [14]. The reaction mixture contained the following in a total of 2 ml, unless otherwise stated: 30 mM G-6-P, 0.1 M maleate buffer (pH 6.7), 4 mM KF; 4 mM EDTA and an appropriate amount of enzyme. Incubation was carried out at 37° for 15–30 min and the reaction was terminated by adding 1 ml cold 10% TCA. One unit of phosphatase activity is defined as 1 μmol substrate hydrolysed/min.

The buffers used to cover the pH range required were 0.1 M HOAc–NaOH (pH 4–5.8), 0.10 M Na maleate (pH 5.8–7), 0.1 M imidazole: NaOH (pH 8) and 0.04 M ethanolamine: HCl (pH 9 and 10). All enzyme activities were measured in duplicate for at least 2 time intervals. Protein was measured by the method of ref. [15] using BSA as a standard. Neutral sugars were measured by the method of ref. [16] using glucose as standard. MW was measured by gel filtration using a Sephadex G-200 column (2 × 120 cm), as described in ref. [17], equilibrated and eluted with 30 mM NaOAc buffer (pH 5) containing 10 mM EDTA, at a flow rate of 15 ml/hr (3 ml fractions). BSA, ovalbumin and trypsinogen were used as protein standards.

Enzyme purification. All purification steps were carried out at 0–4°, unless otherwise stated. *p*-Nitrophenylphosphatase activity was determined at each stage of purification. Scutella (400 g of seeds) of 96 hr seedlings were ground in 0.5 10 mM EDTA (pH 7.4). The homogenate was filtered over gauze and centrifuged at 20 000 *g* for 10 min. The supernatant was fractionated by precipitation with $(NH_4)_2SO_4$ and the protein fraction obtained at 35–60% satn was collected by centrifugation at 20 000 *g* for 15 min, suspended in a small vol. of 10 mM NaOAc buffer (pH 5) and dialysed for 24 hr against 16 l. of the same buffer (with 4 changes of the buffer). The dialysed protein fraction was centrifuged at 20 000 *g* for 10 min to remove precipitated protein and the supernatant containing 82.8 mg protein was applied to a DEAE-cellulose column (2 × 21 cm) previously equilibrated with 10 mM NaOAc buffer (pH 5). Elution was performed with the same buffer at a flow rate of 60 ml/hr (7 ml fractions). The tubes representing the enzyme peak were pooled and dialysed for 24 hr against 16 l. of 20 mM Tris–HCl buffer (pH 7.4) containing 45 mM NaCl (with 4 changes of the buffer). This enzyme fraction, containing 40.5 mg protein, was applied to a DEAE-Sephadex A-50 column (2 × 32 cm) previously equilibrated with the buffer used for dialysis. The column was washed with 1 l. of this buffer to remove unabsorbed protein. The absorbed proteins were eluted with 20 mM Tris–HCl buffer (pH 7.4) containing 0.30 M NaCl at a flow rate of 48 ml/hr (6 ml fractions). The tubes representing the enzyme peak were pooled, dialysed for 24 hr against 4 l. 30 mM NaOAc buffer (pH 5) containing 10 mM EDTA and 4 mM NaF (with 4 changes of the buffer) and concd by ultrafiltration. The concentrate containing 9.0 mg protein was introduced onto a column (2 × 120 cm) of Sephadex G-75 previously equilibrated with the buffer used for dialysis. Elution was performed with this buffer at a flow rate of 20 ml/hr (3 ml fractions). The tubes representing the enzyme peak were pooled and dialysed for 24 hr against 4 l. of 20 mM Tris–HCl buffer (pH 7.4) containing 10 mM Pi (with 4 changes of the buffer). The dialysed fraction containing 4.6 mg protein was introduced onto a column (1 × 20 cm) of DEAE-cellulose previously equilibrated with the buffer used for dialysis and washed with 0.5 l. of this buffer. The absorbed proteins were eluted with 20 mM Tris–HCl buffer (pH 7.4) containing 10 mM Pi and 0.3 M NaCl at a flow rate of 40 ml/hr (4 ml fractions). The tubes representing the enzyme peak were pooled, dialysed 24 hr against 4 l. 10 mM NaOAc buffer (pH 5) with 4 changes of the buffer, concentrated by ultrafiltration and stored at 4°.

Disc electrophoresis was carried out on 7.5% acrylamide gel in Tris–glycine buffer (0.05 and 0.38 M, respectively), pH 8.3, as described in ref. [18]. Protein samples (10–20 μg) were layered in 40% sucrose soln. Bromophenol Blue was used as the tracker dye. A constant current of 3 mA/gel was applied for 2 hr. After the run, the protein bands were located by staining with Coomassie Brilliant Blue. MW was determined by SDS-disc electrophoresis on 10% acrylamide gel containing 0.1% SDS as described in ref. [19]. BSA, ovalbumin and trypsinogen were used as protein standards. The enzyme (36 μg/ml) was incubated for 3 hr, at 37°, with DTNB as described in ref. [20]. The reaction mixture contained in a final vol. of 3 ml, 0.10 M Tris–HCl buffer, pH 8 and 1 mM DTNB. Aliquots (0.1 ml) were taken at appropriate times to measure the *p*-nitrophenylphosphatase activity.

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