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ALKALINE INORGANIC PYROPHOSPHATASE FROM GUAR (*CYAMOPSIS TETRAGONOLOBA*) COTYLEDONS*

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Key Word Index—*Cyamopsis tetragonoloba*; Leguminosae; guar; cotyledons; alkaline inorganic pyrophosphatase; partial purification; properties.

Abstract—Alkaline inorganic pyrophosphatase from guar cotyledons was purified ×110 with about 34% recovery by $(NH_4)_2SO_4$ fractionation, acetone precipitation and chromatography on Sephadex G-200 and DEAE-cellulose. The pH optimum was 8.2 to 8.6. The enzyme was stable only at pH 8.6. Mg^{2+} did not protect the enzyme against inactivation at acid pH. The enzyme showed high specificity for inorganic pyrophosphate (PPi) and acted optimally at 37° and at a PPi concentration of 3.75 mM with a K_m value of 1.65 mM. It required Mg^{2+} for its activity and the optimum Mg^{2+}:PPi ratio for maximum enzyme activity was 1. None of the metal ions tested could replace Mg^{2+}; rather the metal ions and anions were inhibitory. Sodium citrate, sodium cyanide, sodium tungstate and sodium arsenate were the most potent inhibitors of the enzyme. Iodoacetamide did not inhibit the enzyme.

INTRODUCTION

Inorganic pyrophosphatases are now known to be widely distributed in plants [1]. Alkaline inorganic pyrophosphatase (IP) (EC 3.6.1.1), whose activity has been observed in potato tubers [2], sugar cane leaves [3], corn leaves [4, 5], spinach chloroplasts [6–8], corn leaf mitochondria [9] is becoming increasingly interesting. However, not much is known about its behaviour in germinating seeds. Recent studies made by Maslowska and Maslowski [10] have demonstrated the occurrence of this enzyme in the endosperm of germinating corn seeds. While investigating its behaviour and properties in germinating guar seeds, we partially purified this enzyme from cotyledons and various characteristics are reported here.

RESULTS AND DISCUSSION

Partial purification

The enzyme alkaline IP was purified ×110 with *ca* 34% recovery using $(NH_4)_2SO_4$ fractionation, acetone precipitation, Sephadex G-200 and DEAE—cellulose column chromatography (Table 1).

Optimum pH

The activity of IP was measured over a pH range from 2 to 10 using reaction mixtures containing either no Mg^{2+} or 12.5 mM Mg^{2+}. The purified enzyme in the absence of Mg^{2+} was active only at acid pH with an optimum in the range 4.5 to 4.8 (Fig. 1). In the alkaline range, the enzyme required Mg^{2+} for its activity and exhibited pH optima at pH 8.2 to 8.6. Mg^{2+} inhibited activity in the acidic range, the inhibition being about 45% at pH 4.5 and about 72% at pH 6.5. These results clearly indicate that guar cotyledons contain at least two enzymes hydrolysing PPi. One of these (acid IP) exhibits maximum activity at pH 4.5 to 4.8 and does not require Mg^{2+} for its activity, while the other (alkaline IP) acts optimally at pH 8.2 to 8.6 and requires Mg^{2+}. The occurrence of these two enzymes has already been well established in plant tissues [1, 11, 12].

The pH optima in the alkaline range are similar to those reported for IPs from sugarcane [3], corn leaves [4, 5] and corn endosperm [10], but differ slightly from those of potato [2] and spinach chloroplasts [7].

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Table 1. Purification of alkaline inorganic pyrophosphatase from guarcotyledons

Fraction	Volume (ml)	Total protein (mg)	Total activity (nkats)	Specific activity (nkats/mg protein)	Fold purification	Recovery (%)
Crude extract	500	2250	83	0.04	—	100
Ammonium sulphate fraction (40–70%)	110	572	64	0.11	3	77
Acetone fraction	30	258	59	0.23	6	71
Sephadex fraction	60	16.3	37	2.27	57	45
DEAE-cellulose fraction	30	6.6	29	4.39	110	34

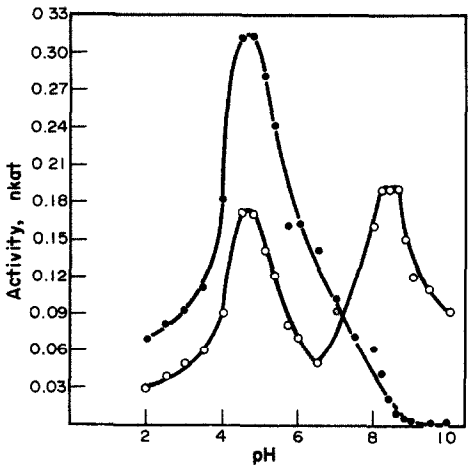

Fig. 1. Pyrophosphatase activity at different pH values in presence and absence of Mg^{2+}.
No Mg^{2+} ●——●; with Mg^{2+} ○——○

The present enzyme differed in its behaviour from that of corn leaves [5], where no activity could be detected in the absence of Mg^{2+} in the pH range 4–10.5, indicating that the enzyme preparation was completely free of Mg^{2+}-inhibited acid IP. However, in the present investigation, the enzyme showed activity in the acidic range in the absence of Mg^{2+}, proving that the degree of purification achieved here was not sufficiently high to exclude acid IP from the enzyme preparation. Similarly, the enzyme preparations obtained from potato [2], sugarcane leaves [3], corn leaves [4] and corn endosperm [10] were also not free from acid IP's.

Effect of buffers, Mg^{2+} and substrate concentration on pH optima

The pH optima remained the same irrespective of the buffer used (the various buffers used were: borate, 0.2 M; borate–NaOH–KCl, 0.05 M; Tris–HCl, 0.05 M and veronal, 0.028 M). However, the activity obtained in Tris–HCl buffer was slightly greater than in the other 3 buffers. This shows that the constituents of the buffers have a slight effect on the activity of this enzyme.

At lower Mg^{2+} concentrations (1.25 and 2.50 mM), the enzyme activity decreased as the pH was raised from 7 upwards, indicating the influence of acid IP. At higher concentrations (5, 12.5 and 25 mM), the activity increased with increase in pH from 7 upwards, with pH optima at 8.2–8.6, which remained unaltered at different concentrations of Mg^{2+}. Similarly, the pH optima remained unaffected by the substrate concentration used. The present findings in respect of Mg^{2+} are contrary to those obtained by El-Badry and Bassham [7], who reported the pH optima to be shifted from 9 at 5 mM Mg^{2+} to 7 at 40 mM Mg^{2+}. Simmons and Butler [4] also observed a shift in pH optima of corn alkaline IP when the Mg^{2+} concentration was lower than the optimum. However, the Mg^{2+} concentration used by these authors was much higher than those employed here.

Effect of pH on stability of the enzyme

To study the effect of pH and any inhibition by the buffer constituents on the enzyme alkaline IP, two sets of tubes containing enzyme were taken. In one set, Mg^{2+} was added before the incubation of the enzyme in the buffer at 37° at different pH values for 2 hr and in the other set, Mg^{2+} was added after the incubation period. The tubes were then cooled and the enzyme activity determined after adding the requisite amount of acid or alkali to bring the solution to the desired pH, buffer, substrate and Mg^{2+}, where necessary.

The enzyme showed maximum stability at pH 8.6. Mg^{2+} did not protect the enzyme against inactivation at acid pH. These results are in conformity with those of Naganna *et al.* [2], who also reported the enzyme to be inactivated at pHs away from the optimum.

Substrate concentrations

The PPi/Mg^{2+} ratio was kept constant while determining the effect of substrate concentrations on enzyme activity. The enzyme activity followed Michaelis–Menten kinetics, and reached a maximum at a substrate concentration of 3.75 mM, above which the activity remained almost constant. The K_m value as determined by Lineweaver–Burk method was 1.65 mM.

Magnesium and pyrophosphate ratio

Full activation of alkaline IP was obtained with 3.75 mM Mg^{2+} in the presence of 3.75 mM PPi, thus giving a ratio of Mg^{2+}: PPi as 1. The higher ratio, even up to 10:1 did not result in any inhibition of the enzyme except that at higher concentrations of Mg^{2+}, a white precipitate appeared in the reaction mixture. This ratio is much lower than those of Bucke [3], Rip and Rauser [5] and Maslowska and Maslowski [10] who obtained a ratio of 4:1, 2:1 and 8:1, respectively for Mg^{2+}:PPi. However, Rip and Rauser [5] showed that even if the ratio is lowered to 1:1, there is little loss in the enzyme activity.

Lambert and Watters [13] suggested that Mg^{2+} and PPi could form 3 complexes: $MgP_2O_7^{2-}$, $Mg_2P_2O_7^0$ and $MgHP_2O_7^{1-}$. The last of these species is pH dependent and its formation requires the protonation of $P_2O_7^{4-}$ before addition to Mg^{2+}. At the pH of our assay, essentially all the PPi is present as $P_2O_7^{4-}$, thus giving rise to both $MgP_2O_7^{2-}$ and $Mg_2P_2O_7^0$ on combination with Mg^{2+}. Since the enzyme was active both at optimum (1:1) and high (10:1) Mg^{2+}:PPi ratios at pH 8.6, both mono-, and di-Mg PPi must be the substrates for the enzyme.

Substrate specificity

The enzyme was highly specific for PPi. None of the substrates such as β-glycerophosphate, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, ATP, ADP, AMP, TPP, diphenyl phosphate and phenyl phosphate was acted upon by the enzyme. This shows that the enzyme preparation was completely devoid of any phosphomonoesterase or phosphodiesterase activity. These results are similar to those reported earlier [5, 7, 10].

Metal ion specificity

To study the effect of various metal ions, two types of experiments were conducted. In one set of the experiment, Mg^{2+} was included in the reaction mixture along with the metal ion under test, while in the other set of experiment, Mg^{2+} was excluded.

Table 2. Effect of metal ions on alkaline inorganic pyrophosphatase

Metal ion	Ion concentration (mM) 2.5	6.25	12.5	25
	Activity (% of control)			
Cd^{2+}	64	17	5	3
Zn^{2+}	71	50	9	5
Cu^{2+}	62	22	9	7
Hg^{2+}	97	84	37	17
Ni^{2+}	22	18	5	4
Co^{2+}	53	29	10	4
Mn^{2+}	42	22	7	4
Fe^{3+}	100	56	7	4
Ca^{2+}	62	26	9	4
Ba^{2+}	75	37	7	4
Al^{3+}	100	61	5	4
Na^{+}	100	100	100	73
K^{+}	100	100	100	75
Ag^{+}	100	71	5	4

Table 4. Effect of inhibitors on alkaline inorganic pyrophosphatase

Inhibitor	Inhibitor concentration (mM) 1	2	5	10
	Activity (% of control)			
Potassium thiocyanate	99	43	5	—
Sodium oxalate	43	13	4	—
Sodium citrate	2	—	—	—
Sodium succinate	99	57	18	—
Potassium tartrate	99	60	14	—
Sodium cyanide	2	—	—	—
Urea	84	48	5	—
Thiourea	91	44	3	—
Sodium molybdate	76	48	9	—
Sodium tungstate	3	—	—	—
Ammonium thiocyanate	94	44	3	—
EDTA	44	14	3	—
Sodium arsenate	2	—	—	—
Iodoacetamide	107	100	98	98

The enzyme showed an absolute requirement for Mg^{2+}. No activity was observed with any other mono-, di- or polyvalent metal ions tested. In the presence of Mg^{2+}, the various metal ions except Na^{+} and K^{+} inhibited the enzyme (Table 2). At higher concentrations, Na^{+} and K^{+} were also inhibitory. Cysteine could not reverse the inhibition caused by these metal ions: EDTA reversed the inhibitory effects, but its effect was variable. In respect of Mg^{2+} requirement, the present enzyme therefore resembles those specific IPs which have been reported to be present in yeast [14], liver, muscle, heart, lung, kidney, spleen, intestine, prostate and testes [15], erythrocytes [16], and higher plants [1–10].

Effect of anions

In testing the effect of different anions on enzyme activity, Mg^{2+} was always present in the reaction mixture. Only F^{-} had a pronounced inhibitory effect on the enzyme (Table 3). Its inhibition was striking even at the lowest concentration (6.25 mM). SO_4^{2-} also partially inhibited the enzyme at lower concentrations. At the highest concentration, it completely inhibited the enzyme, while the others, viz. Cl^{-}, Br^{-}, I^{-}, and NO_3^{-} could inhibit the enzyme only up to the extent of 29%. The inhibition by F^{-} may be attributed to the prevention of Mg^{2+} activation of enzyme due to the low solubility product of MgF_2.

Inhibitors

Sodium citrate, sodium cyanide, sodium tungstate and sodium arsenate, known earlier to inhibit plant acid and alkaline IPs [2, 3, 12] were the most potent inhibitors of the guar enzyme (Table 4). The enzyme was also inhibited by sodium oxalate, urea, sodium molybdate, EDTA, potassium thiocyanate, sodium succinate, potassium tartrate, thiourea and ammonium thiocyanate. However, the concentration required to inhibit the enzyme was comparatively more in these cases than the first 4 inhibitors.

Iodoacetamide did not inhibit the enzyme even at concentrations as high as 10 mM. This indicates that the enzyme does not require thiol groups for its activity. Lack of activation by cysteine and glutathione further confirmed these findings.

In the absence of any information about the nature of the chemical groups involved in the active site of the enzyme alkaline IP, it is very difficult to explain at this stage the exact mechanism of action of the dicarboxylic acid ions. However, these are the general non-specific inhibitors of this enzyme and based on the charge on the ion, it could be said that they might also be acting chaotropically in a manner similar to that of thiocyanates. Alternatively, they may be capable of binding metal ions thus making Mg^{2+} unavailable to the enzyme. The only definite conclusion which could be drawn from the present inhibitor studies is that the –SH groups are not involved in the action of this enzyme.

Table 3. Effect of anions on alkaline inorganic pyrophosphatase

Anion	Anion concentration (mM) 6.25	12.5	25	250
	Activity (% of control)			
Cl^{-}	94	100	94	71
Br^{-}	100	100	100	71
I^{-}	100	101	100	71
F^{-}	3	3	4	—
SO_4^{-2}	57	50	37	—
NO_3^{-}	102	104	100	71

EXPERIMENTAL

Materials. Cotyledons from guar seedlings, germinating in H_2O in Petri dishes at 30° for 5 days served as a source of the enzyme.

Enzyme purification. All the purification steps were carried out at 4°. The crude extract was prepared by homogenizing 50 g cotyledons in small lots in a chilled mortar and pestle with acid washed sand using 10 ml of 28 mM veronal buffer (pH 8.6) per g of tissue. The homogenate was centrifuged for 30 min at 10000 g and the supernatant made to 500 ml with cold veronal buffer, which was then purified by fractionation with $(NH_4)_2SO_4$. The main portion of alkaline IP activity pptd between satn levels of 40 and 70%. This fraction was collected by centrifugation and after dissolving in cold veronal buffer, was dialysed for 24 hr against the same buffer (diluted, 1:10)

with repeated changes. To this fraction at −10°, cold Me_2CO was added to a concn of 150%, the soln stirred for few min and the ppt. collected by centrifugation. This fraction was then dissolved in cold veronal buffer and chromtographed on a Sephadex G-200 column (60 × 3 cm; flow rate 9 ml/hr) that had been equilibrated with the same buffer; elution was repeated with the veronal buffer. The active fractions (30–50 of 3 ml each) were pooled and further purified by passing through a DEAE-cellulose column (40 × 2 cm). A gradient was established with increasing concn of NaCl (0.25–0.6 M) prepared in the veronal buffer. Fractions (3 ml) were collected at a flow rate of 1.5 ml/min; the enzyme was eluted at 0.35–0.4 M NaCl concn. The eluate fractions containing the active enzyme were combined and this enzyme prepn was used for all expts reported here.

Enzyme assays. Alkaline IP was assayed in a vol. of 4 ml containing in μmol: $MgCl_2$, 50; $Na_4P_2O_7$, 15; veronal (pH 8.6), 50; and 0.5 ml enzyme. Following incubation at 37° for 15 min, 1 ml 20% TCA was added, the mixture kept at low temp. for 15 min and then centrifuged for 5 min at 8000 g. The Pi obtained in the supernatant was then determined according to ref. [17]. Activity is expressed in nkat. Protein was estimated according to ref [18] using crystalline BSA as standard.

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