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PARTIAL PURIFICATION AND SOME PROPERTIES OF ALKALINE INORGANIC PYROPHOSPHATASE FROM *ZEA MAYS* LEAVES*

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Abstract—Alkaline inorganic pyrophosphatase from corn leaves has been purified 1750-fold by fractionation with $(NH_4)_2SO_4$ and $Ca_3(PO_4)_2$ gel followed by DEAE-cellulose column chromatography. The enzyme which chromatographed as a single peak on DEAE-cellulose, required Mg^{2+} for activity at a pH optimum of 8·3–8·8. The preparation was devoid of Mg^{2+} inhibited acid pyrophosphatase. The enzyme exhibited a high specificity for inorganic pyrophosphate as substrate to the virtual exclusion of several phosphorylated organic compounds. Both mono- and di-magnesium pyrophosphate were substrates for the enzyme, the greater activity occurring with the mono-magnesium salt. The enzyme was inhibited by fluoride and several divalent cations excepting Cu.

INTRODUCTION

WE HAVE investigated the role of alkaline inorganic pyrophosphatase (E.C. 3.6.1.1) in chromatin-directed RNA synthesis. For this purpose, we required a preparation of relatively high purity. Available purification procedures for inorganic pyrophosphatase from plant tissues[1, 2] do not yield preparations of high specific activity and apparent homogeneity. We report here a procedure for the partial purification of alkaline inorganic pyrophosphatase from corn leaves and some of the properties of the enzyme preparation.

RESULTS AND DISCUSSION

Partial Purification of Alkaline Inorganic Pyrophosphatase from Corn Leaves

The results of a typical experiment are shown in Table 1 where a purification of 1750-fold was obtained with an 11 per cent recovery. No comparable data are available for previous preparations of the enzyme from plant material.[1, 2]

The behaviour of corn alkaline inorganic pyrophosphatase on $(NH_4)_2SO_4$ fractionation resembled human erythrocyte pyrophosphatase[3] in that the bulk of the enzyme was recovered in the 0·35–0·53 g/ml starting volume fraction. Corn alkaline inorganic pyrophosphatase differed markedly however, from potato pyrophosphatase where 65 per cent was recovered in the 0·224–0·448 g/ml starting volume fraction.[1]

Nearly 50 per cent of the protein but little alkaline inorganic pyrophosphatase was adsorbed to $Ca_3(PO_4)_2$ gel up to a gel–protein ratio of 1:5. The fraction between gel–protein ratios of 1·5:5 and 3·5:5 adsorbed 70 per cent of the total enzymatic activity and only 35 per cent of the protein. Elution of this $Ca_3(PO_4)_2$ gel fraction with buffer of low

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TABLE 1. PURIFICATION OF CORN LEAF ALKALINE INORGANIC PYROPHOSPHATASE

Treatment	Volume (ml)	Total protein (mg)	Total units*	Specific activity (units/mg protein)	Recovery (%)	Purification (fold)
Crude extract	1000	23,890	8056	0·34	100	—
0·35–0·53 g/ml $(NH_4)_2SO_4$	67	184·6	6380	34·56	79·2	104
Calcium phosphate gel	8·0	8·41	1430	170·03	17·7	504
DEAE-cellulose	8·0	1·51	894	592·05	11·1	1750

* 1 unit = 1·0 μmole Pi/min.

ionic strength (up to 80 mM Tris-HCl) removed 40 per cent of adsorbed protein and little pyrophosphatase. Three elutions with increasing concentrations of NaCl in buffer allowed a recovery of 63 per cent of the pyrophosphatase activity adsorbed initially. Although the adsorption profile remained constant with gel age, the recovery of enzyme from $Ca_3(PO_4)_2$ gel decreased progressively with gel age beyond 2 weeks.

Alkaline inorganic pyrophosphatase activity was eluted as a single peak from a DEAE-cellulose column. The leading and trailing 8 ml fraction represented 4·5 and 9·1 per cent of the activity in the single peak fraction respectively (Table 1). Butler and Bennett[4] reported 2 peaks of alkaline inorganic pyrophosphatase activity in crude corn leaf extracts on DEAE-cellulose columns. By comparing our elution profiles, we inferred that the single peak present in our partially purified preparation represented the first of their two peaks.

We employed 2 other techniques in the purification of alkaline inorganic pyrophosphatase but without success. The butanol fractionation of Morton[5] resulted in nearly complete inactivation. Bacterial pyrophosphatases have been adsorbed to columns of Mg-pyrophosphate on a support of coarse grade Sephadex G-25,[6] but dialyzed $(NH_4)_2SO_4$ fractionated corn alkaline inorganic pyrophosphatase did not adsorb to such Mg-pyrophosphate columns operating at 4° or 26°.

Optimum pH

For the pH study only, the 196 μmoles Tris-HCl in standard assays were augmented with 196 μmole each of citric acid, imidazole and glycine with HCl or NaOH added to give the desired pH. The pH optimum for alkaline inorganic pyrophosphatase, purified through to the DEAE-cellulose step, was 8·3–8·8 in the presence of 10 mM Mg^{2+}. In the absence of Mg^{2+} no pyrophosphatase activity was detected from pH 4·0–10·5, indicating that the preparation was free of the Mg^{2+} inhibited acid pyrophosphatase prevalent in plant tissues.[1, 7] Simmons and Butler[2] reported a pH optimum between 8 and 9 for pyrophosphatase in crude extracts of corn in the presence of 10 mM Mg^{2+}. However, at 2·5 and 1·0 mM Mg^{2+} the optimum shifted to pH 9·0. In comparison to our results, the pH curves obtained by Butler and Bennett[4] for crude corn extract may be the result of an acid inorganic pyrophosphatase inhibited by Mg^{2+} and 2 Mg^{2+} activated alkaline inorganic

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pyrophosphatases. The enzyme from corn differs markedly from the alkaline pyrophosphatase of potato,[1] which has a pH optimum of 9–10 and is precipitated by considerably less $(NH_4)_2SO_4$.

Magnesium and Pyrophosphate Concentration and Ratio

Full activation of alkaline inorganic pyrophosphatase, purified through to DEAE-cellulose chromatography, was obtained with 10 mM Mg^{2+} in the presence of 1·0 mM $Na_4P_2O_3$. 2·0 mM Mg^{2+} gave 97 per cent of full activation, while excesses up to 60 mM resulted in relatively small inhibition (25%). The partially purified pyrophosphatase used here was activated to a higher degree with a given amount of Mg^{2+} than was the crude enzyme preparation of Simmons and Butler.[2] However, more Mg^{2+} was required for full activation of our enzyme preparation than by alkaline pyrophosphatases from yeast[8] and firefly[9] where 1 mM Mg^{2+} sufficed.

The optimal pH and optimum Mg^{2+}:pyrophosphate ratio provide information for identifying the natural substrate. In the presence of 1 mM pyrophosphate, the optimum Mg^{2+}:pyrophosphate ratio was 2:1. At the lower ratio of 1:1 and the higher ratio of 60:1, essentially 75 per cent of maximum activity was still present. Lambert and Watters[10] suggested that Mg^{2+} and pyrophosphate 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 as its formation requires the protonation of $P_2O_7^{4-}$ before addition to Mg^{2+}. At the pH of our assays, essentially all the pyrophosphate 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 alkaline inorganic pyrophosphatase was active both at low (1:1) and high (60:1) Mg^{2+}: pyrophosphate ratios at pH 8·7, both mono- and di-magnesium pyrophosphate must be substrates for the enzyme. The latter salt may be acted upon at a slower rate since activity was only 75 per cent of maximum at the highest Mg^{2+}: pyrophosphate ratio. Pynes and Younathan[3] reached the same conclusion regarding the activity at pH 7·7 of human erythrocyte pyrophosphatase.

Substrate Specificity

Alkaline inorganic pyrophosphatase purified through to DEAE-cellulose chromatography was exposed to 1 mM concentrations of phosphorylated compounds in the absence of pyrophosphate of otherwise standard assays. Sodium pyrophosphate was by far the most active substrate for the enzyme. Fructose-1,6-diphosphate and ATP were acted upon to the extent of 1·26 and 0·47 per cent of the rate observed for pyrophosphate respectively. The enzyme preparation was not active towards AMP, ADP, NADP, glucose-1-phosphate and glucose-6-phosphate. Similar reactions conducted at pH 6·0 with and without Mg^{2+} showed no hydrolysis, indicating that the preparation was devoid of acid pyrophosphatase activity found in crude corn extracts.[2] Partially purified alkaline inorganic pyrophosphatase from erythrocytes[3] and yeast[11] also showed a high specificity for inorganic pyrophosphate as substrate.

Metal Ion Activation

Of all metals tested up to 3·0 mM concentration Mg^{2+} was the most efficient activator of alkaline inorganic pyrophosphatase (Table 2). The highest activation by Mn^{2+} was only 7 per cent that with 3 mM Mg^{2+}. Pynes and Younathan[3] showed that Mn^{2+} and Co^{2+} activated human erythrocyte pyrophosphatase only 7 per cent as much as did Mg^{2+}.

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TABLE 2. METAL ION ACTIVATION OF PARTIALLY PURIFIED CORN ALKALINE INORGANIC PYROPHOSPHATASE*

Cation concentration (mM)	Specific activity for cation (units†/mg protein)						
	Mn	Ni	Co	Fe	Zn	Cu	Mg
0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
0·12	0·0	4·0	0·0	11·25	17·25	0·0	0·0
0·30	16·25	4·0	0·0	ppt.‡	0·50	1·75	5·25
0·80	42·25	0·0	14·5	ppt.	0·0	6·25	150
1·80	2·75	0·0	10·5	ppt.	0·0	8·75	548
3·00	ppt.	0·0	0·0	ppt.	ppt.	ppt.	553

* The metals were used as their chlorides in the standard assay lacking Mg^{2+}. The enzyme preparation used was that following DEAE-cellulose column chromatography and dialysis against 1 mM mercaptoethanol, 50 mM Tris-HCl (pH 8·7). A volume equivalent to 0·26 units of pyrophosphatase activity prior to dialysis was added to each reaction tube.

† 1 unit = 1·0 μmole Pi/min.

‡ ppt. = incipient precipitation during incubation.

The enzyme activity measured in 3 mM Mg^{2+} (Table 2) was 84 per cent of the activity present prior to dialysis. For the purposes of this experiment, dialysis in the absence of Mg^{2+} was short and the preparation was used immediately. On prolonged dialysis in the absence of Mg^{2+}, pyrophosphatase could not be reactivated. Routinely, no loss of pyrophosphatase activity occurred when the preparation was exposed to Mg^{2+} during all manipulations, even storage as a concentrate at −15° for 2 months.

Inhibitors

The divalent metal cations Co^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+} were the most effective inhibitors of corn alkaline inorganic pyrophosphatase (Table 3). Relatively high concentrations

TABLE 3. THE EFFECT OF SELECTED METALS AND NON-METALS ON CORN ALKALINE INORGANIC PYROPHOSPHATASE*

Chemical	Concentration (M)	% Inhibition
$MgCl_2$	2×10^{-2}	9
	6×10^{-2}	25
$MnCl_2$	6×10^{-4}	47
	1×10^{-3}	75
$CoCl_2$	2×10^{-4}	30
	2×10^{-3}	58
$CaCl_2$	2×10^{-4}	49
	2×10^{-3}	84
$ZnCl_2$	2×10^{-4}	48
	2×10^{-3}	99
$CuCl_2$	2×10^{-3}	1
NaF	5×10^{-4}	93
NH_4SCN	1×10^{-2}	0
$(NH_4)_2SO_4$	5×10^{-3}	9
HCHO	2×10^{-2}	0

* Activity of DEAE-cellulose purified enzyme was tested in the standard assay medium supplemented with the selected chemicals to the final concentrations. The reference was the standard assay.

of Mg^{2+} gave small inhibitions. In contrast to the potato alkaline pyrophosphatase,[1] the corn enzyme was not inhibited by 2 mM Cu^{2+}. Of the non-metals tested, fluoride was the most effective inhibitor, while formaldehyde, cyanide and ammonium sulfate were poor inhibitors. Naganna *et al.*[1] found formaldehyde to inhibit potato alkaline pyrophosphatase, apparently by formylating free amino groups required for activity. By comparison, corn alkaline inorganic pyrophosphatase activity does not appear to depend on free amino groups. The growth regulators indole acetic acid, benzyladenine and gibberellic acid at concentrations of 11·4, 5 and 10 μM respectively, did not inhibit or promote corn alkaline inorganic pyrophosphatase *in vitro*.

EXPERIMENTAL

Plant material. Corn (*Zea mays* L., var. United Hybrid 108) seeds, planted in vermiculite, were grown in a greenhouse supplemented with artificial illumination for a 13-hr photoperiod. Seven days after planting, the shoots were cut off at the base of the first leaves and the tissue frozen.

Enzyme extraction and partial purification. Small lots of frozen corn tissue were ground with a small amount of acid washed sand in a chilled mortar and pestle using 10 ml of 50 mM Tris-HCl (pH 8·7) per g of tissue. The homogenate was pressed through 4 layers of cheesecloth, adjusted to pH 8·7 and centrifuged at 11,000 *g* for 15 min. These and subsequent procedures were carried out at 4°.

100 ml of cleared homogenate was treated with 35 g finely ground $(NH_4)_2SO_4$ and the precipitate discarded following centrifugation at 21,000 *g* for 20 min. The supernatant was then supplied with a further 18 g $(NH_4)_2SO_4$ and the precipitate collected by centrifugation. This $(NH_4)_2SO_4$ fraction was dissolved in 50 ml 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 8·7) and dialyzed against three 2-l. changes of the same buffer. Following clarification by centrifugation, the pH of the dialysate was adjusted to 7·9.

Two week old calcium phosphate gel, prepared according to Colowick,[12] was added in the ratio of 1·5:5 (mg dry weight of gel:mg protein initially present) to the above dialysate. The mixture was stirred for 15 min and the sediment discarded following centrifugation (10 min at 10,000 *g*). The supernatant was treated with calcium phosphate gel to a ratio of 3·5:5 and the precipitate collected by centrifugation. Protein having little alkaline inorganic pyrophosphatase activity was removed from this gel fraction by successive elutions with 20, 40 and 80 mM Tris-HCl (pH 9·0) containing 10 mM $MgCl_2$. The gel was stirred in eluant for 1 hr followed by sedimentation of the gel and reextraction in the stronger buffer. Alkaline inorganic pyrophosphatase was then removed from the gel with 0·34, 0·60 and 0·86 M NaCl in 10 mM $MgCl_2$, 250 mM Tris-HCl (pH 9·0). Equilibration with eluant was for 3 hr in each case. The NaCl-buffer eluates were pooled, dialyzed against two 2-ml. changes of 10 mM $MgCl_2$, 50 mM Tris-acetic acid (pH 7·65) and concentrated by allowing dry Sephadex G-25 to swell in the solution.

The enzyme preparation was then chromatographed on a DEAE-cellulose column essentially according to Butler and Bennett.[4] The column was eluted with a linear Mg^{2+} gradient by mixing 500 ml each of 20 mM and 240 mM $MgCl_2$ in 50 mM Tris-acetic acid (pH 7·65). 8 ml fractions were collected at a flow rate of 0·8 ml/min.

Enzyme assays. Alkaline inorganic pyrophosphatase was measured in a 5·0 ml reaction volume containing in μmole: $MgCl_2$, 50; $Na_4P_2O_7$, 5; Tris-HCl (pH 8·7), 196; and 0·5 ml enzyme suitably diluted to give no more than 80% hydrolysis of the pyrophosphate. Following incubation at 37° for 15 min, 2·5 ml ice-cold 10% (w/v) trichloroacetic acid was added, the mixture kept at 0° for 15 min and then centrifuged for 5 min at 8000 *g*. The entire supernatant was then assayed for Pi according to Allen.[13] One unit of alkaline inorganic pyrophosphatase activity is the amount of enzyme which causes the net appearance of 1·0 μmole Pi/min under the assay conditions described. Protein was estimated spectrophotometrically[14] or according to Lowry *et al.*[15] using crystalline bovine serum albumin as standard.

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