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# MAIZE LEAF INORGANIC PYROPHOSPHATASE: ISOZYMES, SPECIFITY FOR SUBSTRATES, INHIBITORS, AND DIVALENT METAL IONS, AND pH OPTIMA

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## SUMMARY

1. Maize leaf inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) exists as two major isozyme forms which are readily separable on polyacrylamide gel electrophoresis but are difficult to resolve by ion-exchange chromatography or isoelectric focusing. All 25 mutants examined had the same isozyme set; in 20 of these Isozyme 2 predominated and in the remaining five approximately equal amounts of Isozymes 1 and 2 were present.

2. Only Isozyme 2 is present in extracts of very young etiolated plants. Isozyme 1 could be obtained free of Isozyme 2 from light-grown seedlings treated with cycloheximide.

3. Isozymes 1 and 2 appeared to be similar in size and enzymatic properties, but thermal denaturation and immunological studies indicated that they have significant differences in structure.

4. These maize leaf pyrophosphatase isozymes and analogous enzymes from other plants have potent activity at pH 6.2 using $Zn^{2+}$ as the activating metal ion, as well as their previously reported high activity at pH 9.0 with $Mg^{2+}$.

5. Although leaf pyrophosphatase appears to be an integral part of the $C_4$ pathway of $CO_2$ fixation, metabolites of that pathway apparently have no regulatory effect upon the enzyme.

## INTRODUCTION

We have previously described a potent inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) from maize leaves[1] and demonstrated that its synthesis is under phytochrome control in the developing plant[2]. This enzyme has subsequently been partially purified by Rip and Rauser[3]; enzymes with similar properties have been purified from sugar cane[4] and from spinach chloroplasts[5,6].

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Our present studies are an extension of our previous finding of two major forms, presumably isozymes[7], of this leaf enzyme[2]. In particular, we have investigated possible differences in physical properties of the two forms, and have attempted to determine their intracellular location, susceptibility to metabolic control, and relative distribution in maize mutants. We have also made additional observations on the specificity and pH optima of this enzyme system.

## MATERIALS AND METHODS

ATP, ADP, pyruvate and cycloheximide were obtained from Calbiochem; L-malic acid, oxaloacetic acid, AMP, phosphoenolpyruvate, chloramphenicol and cacodylic acid were obtained from Sigma Chemical Company, St. Louis.

Field hybrid maize seeds (variety Migro M-540) used in all routine studies were a gift from the Walter J. Harpel Seed Company, Crawfordsville, Indiana. Maize mutants were obtained from the Maize Genetics Coop at the University of Illinois. All seeds were soaked overnight in deionized water before planting. Light-grown seedlings were grown in vermiculite for 7 days (some mutants were grown longer) under constant illumination from a Champion 150 W incandescent reflector lamp 1 m above the plants. Etiolated seedlings were grown in light-tight canisters as previously described[2].

In many cases the leaf tissue was stored frozen; no consistent qualitative or quantitative difference was observed in the enzymes obtained from fresh and frozen tissue. Homogenization was for 1 min in an ice-chilled Waring Blendor, using 10–40 ml of extraction buffer per g fresh weight of leaf tissue. Most of the extracts were prepared using as the extraction medium a 10% (w/v) suspension of Dowex 1-X8 (Cl) anion-exchange resin (analytical grade, from BioRad Laboratories, Richmond, Calif.)[8] in 0.01 M Tris–HCl, pH 8.0. Some of the later extracts were prepared in 25% (v/v) glycerol and 10 mM $MgCl_2$ in 0.02 M Tris–HCl, pH 7.4; extracts in this buffer had similar amounts of activity but enhanced stability of isozyme patterns. After filtration through nylon tulle, the extract was centrifuged at 10 000 $\times$ $g$ for 15 min at 3 °C. The supernatant fluid was decanted and assayed; loss of alkaline pyrophosphatase activity was negligible over a period of several days at 3 °C.

Standard pyrophosphatase assays at pH 9.0 were done as previously described[1]. For measurement of acid pyrophosphatase activity, 1.0 ml of ammonium cacodylate, pH 6.2, containing 2 mM $ZnCl_2$ and appropriate amounts of enzyme, was warmed to 30 °C before the reaction was started by addition of 10 $\mu$l of 0.1 M $Na_4P_2O_7$ (final $[PP_i] = 1$ mM). On standing several hours a precipitate, presumably zinc pyrophosphate, is formed in this assay mix. Product $P_i$ was measured as previously described[1]. One unit of pyrophosphatase activity is that amount of enzyme which hydrolyzes 1 $\mu$mole of $PP_i$ per min in the standard assay at pH 9.0.

Analytical polyacrylamide gel electrophoresis was run on 7% acrylamide, 0.186% *N,N'*-methylenebisacrylamide (both from Eastman Organic Chemicals) gels at pH 8.9[9]. Crude extract containing 0.02 to 0.03 units of activity (10–50 $\mu$l) was electrophoresed at 3 mA per gel. Pyrophosphatase activity on the gels was detected by a method similar to that reported by Tono and Kornberg[10]. After removal of the portion of the gel below the bromophenol blue marker dye, the gels were incubated 5 min at 30° C submerged in the standard pH 9.0 assay mix, rinsed with water,

and the activity visualized as distinct white bands on a clear background by precipitation of the product $P_i$ by immersion in the triethylamine–molybdate reagent which is specific for $P_i$[11]. $R_F$ values for activity bands were calculated with respect to distance travelled by the marker dye.

## RESULTS AND DISCUSSION

### *Evidence for isozymes*

Aliquots of crude leaf extracts of standard maize hybrids subjected to polyacrylamide gel electrophoresis and then assayed for pyrophosphatase activity on the gel developed a light activity band at $R_F \approx 0.5$ (designated Isozyme 1), a denser band of activity at $R_F \approx 0.4$ (Isozyme 2), and a very faint diffuse smear at $R_F \approx 0.6$–0.8. The latter may be an artifact of the electrophoretic system; it has been ignored in this work. Because of its convenience, this technique has been routinely used to monitor the isozyme distribution after various treatments.

The previously demonstrated resolution of the enzyme into two forms by chromatography on DEAE-cellulose[2] has been found to vary considerably with different maize hybrids. A typical chromatographic elution pattern obtained with the standard hybrid (Migro M-540) employed in these studies (the hybrid utilized in the previous work was no longer available) is shown in Fig. 1. Resolution into two peaks was incomplete, and polyacrylamide gel electrophoresis showed that both isozymes were present in varying amounts in all fractions. Alteration of pH or steepness of the $MgCl_2$ gradient, inclusion of reducing agents, or pre-treatment of the extract by gel filtration on Sephadex G-50 did not improve the resolution.

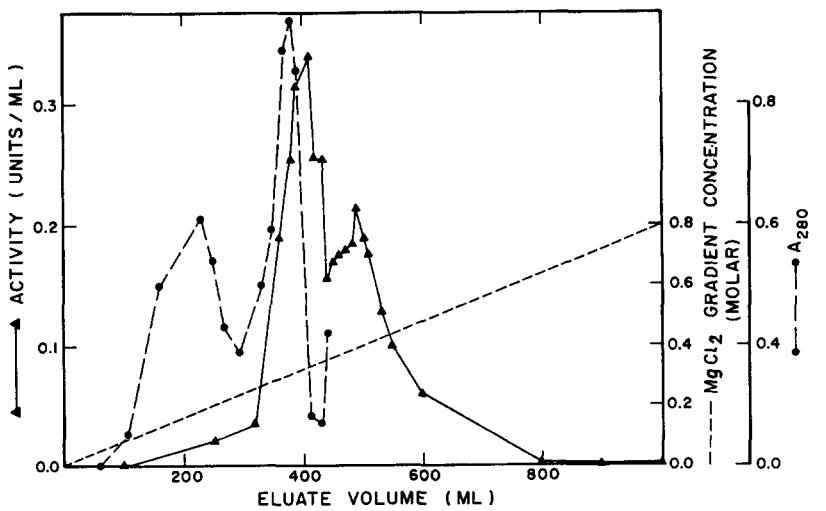


Fig. 1. DEAE-cellulose column chromatography of maize alkaline pyrophosphatase. The extract from 5 g of light-grown leaves (15 ml, 67.5 units) was applied to a 2.5 cm × 25 cm column of DEAE-cellulose (Mann Research Laboratories) which had equilibrated with 0.05 M Tris–HCl buffer, pH 7.65. The column was eluted with a linear gradient of 0 to 0.08 M $MgCl_2$ in 0.05 M Tris–HCl buffer, pH 7.65 (500 ml of each). Fraction size was 10 ml and flow rate was 2 ml/min. 71% of the applied alkaline pyrophosphatase activity was recovered. Protein concentration was estimated by absorbance at 280 nm. The chromatography was carried out at 7 °C.

Similar difficulty in resolving the isozymes was encountered using preparative gel electrophoresis and isoelectric focusing (Fig. 2). Both isozymes appear to have isoelectric points near 5.0.

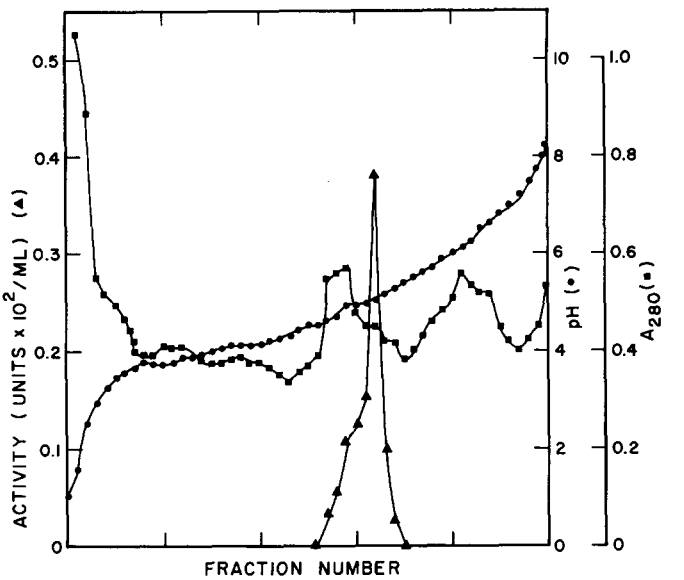


Fig. 2. Isoelectric focusing of maize alkaline pyrophosphatase. The extract from 7 g of light-grown leaves (23 ml, 83.5 units) was electrofocused in pH 3–6 ampholyte for 36 h at 250 V, in a 110 ml LKB isoelectric column set up according to the manufacturer's direction. The apparatus used was the LKB 8121 gradient mixer (LKB producter AB, Bromma, Sweden). The anode solution (bottom electrode) contained 0.2 ml of $H_2SO_4$ and 12 g of sucrose in 14 ml distilled water and the cathode solution (upper electrode) contained 0.1 g of NaOH in 10 ml distilled water. For preparation of the gradient, the dense solution contained 1.875 ml of 40% ampholine type 8141 and 28 g of sucrose in 42 ml distilled water. The light solution contained 0.625 ml of 40% ampholine and the crude extract made up to 60 ml with distilled water. After 36 h at 250 V, 4 °C, 1-ml fractions were collected and assayed for alkaline pyrophosphatase activity (▲), absorbance at 280 nm (■), and pH (●). 60% of the applied pyrophosphatase activity was recovered.

*Isozyme patterns in maize mutants*

In an attempt to find a mutant strain which contains only one of the isozymes and thus obviate the necessity for resolving the usual isozyme pair, the isozyme patterns of 25 maize mutants were examined by gel electrophoresis. Mutants were selected for chlorophyll deficiency or some related abnormality because the intracellular location of at least part of the leaf inorganic pyrophosphatase is in chloroplasts[1,4], and because the enzyme has been implicated in photosynthetic $CO_4$ fixation[2,12], although its synthesis has been shown to be independent of chlorophyll synthesis[1]. Most of the mutants showed the normal isozyme pattern observed in standard hybrids. The five anomalous patterns did not lack either of the two bands, and no new bands were present; they were distinguished from the normal patterns by the approximately equal intensity of the two bands. Of these five variants, four are albino plants with only 20–40% as much activity as green plants; the single exception is a yellow-green mutant, $yg_2$, which had the same level of activity as controls. Because

all mutants tested contained both isozymes, the problem of the resolution of the isozymes was approached in another way.

*Preparation of the isozymes separately*

In agreement with our previous suggestion that one of the isozyme forms is light-induced and the other is constitutive[2], it was found that extracts of etiolated seedlings up to 3 days old contained only one form, Isozyme 2. This observation provided a convenient method for obtaining this isozyme free of Isozyme 1. Extracts of somewhat older etiolated seedlings contained appreciable amounts of Isozyme 1, in agreement with our previous finding that other factors can eventually cause the formation of the isozyme normally induced by light[2].

Isozyme 1 was obtained free of Isozyme 2 from plants which had been treated with cycloheximide. We had previously suggested that one of these forms of the enzyme may originate in the chloroplasts and the other in the cytoplasm[2]. In an effort to obtain evidence on this point we have utilized a variety of treatments with cycloheximide and chloramphenicol, which inhibit cytoplasmic and chloroplast protein synthesis, respectively[13,14]. We have observed complex effects which depend upon a variety of factors including the method of application of the inhibitor, so that it has not been possible to unequivocally determine the intracellular origin of the enzyme forms using this inhibitor technique. However, in maize seedlings sprayed three times daily with $10^{-4}$ M cycloheximide in 1% Triton X-100, Isozyme 2 is not detectable after three days of antibiotic treatment. This is consistent with our earlier suggestion that one of the isozyme forms "turns over" and that its resynthesis is inhibited by cycloheximide[2]. Whether or not this explanation applies to the system of pyrophosphatase isozymes as it does to catalase isozymes in maize seedlings[15], the observation provides a useful method for obtaining plant extracts which contain only Isozyme 1.

The existence of extracts which separately contain only Isozymes 2 and 1 rules out the possibility that the isozyme pattern observed on gel electrophoresis is an artifact of the technique. Mixtures of extracts which separately contained the two isozymes produced the normal double banded pattern of enzyme activity on the gel.

*Comparison of isozyme properties*

Properties of the two isozymes in separate extracts prepared as described above were compared in several ways. Fig. 3 presents a representative heat denaturation study; the results indicate that Isozyme 2 is the more stable of the two at 60 °C.

Rabbit antisera to crude extracts containing each of the isozymes separately demonstrated qualitative differences in the proteins of the preparations as determined by Ouchterlony double-diffusion tests[16]. Because of the impure nature of the preparations, it was not possible to relate these differences in protein components to the pyrophosphatase isozyme system. Representative quantative data on isozyme inhibition by antisera are presented in Fig. 4; analogous results were observed with varied regimes for exposure of the enzymes to antisera before assay. The isozymes appear to be immunologically cross-reactive; both forms were inhibited by antisera prepared against the other. However, they are not identical; Isozyme 2 was consistently more susceptible to inhibition than was Isozyme 1, even to antisera against Isozyme 1.

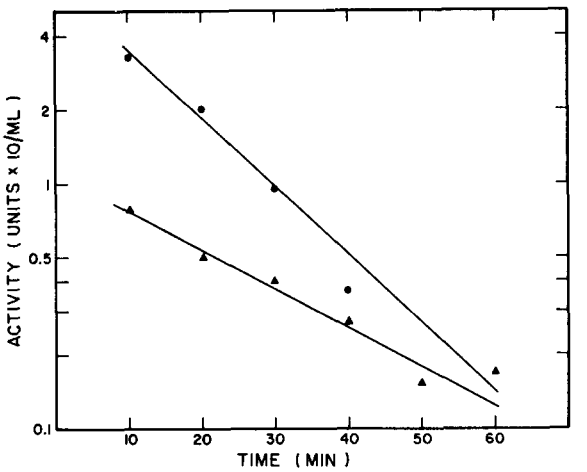


Fig. 3. Thermal denaturation of pyrophosphatase isozymes. Extracts containing the isozymes separately were obtained as described in the text utilizing the Dowex 1-X8-containing buffer. On heating at 60 °C a large protein precipitate formed within 5 min and was removed by decantation of the supernatant fluid. The denaturation data was obtained using the soluble protein fraction which contained most of the original pyrophosphatase activity. At specified times, 1-ml aliquots were removed, stored in ice, and assayed at the completion of the heat treatment. Isozyme 1, (●); Isozyme, 2 (▲).

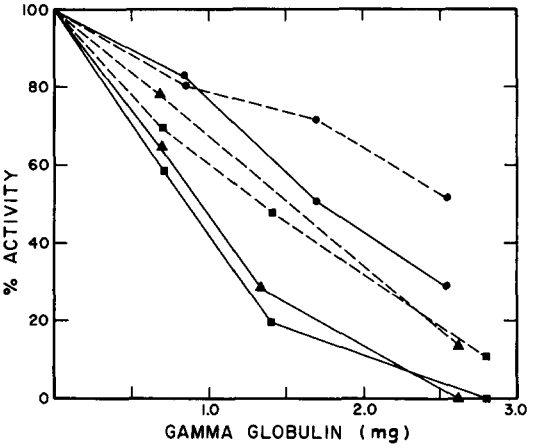


Fig. 4. Antisera inhibition of pyrophosphatase isozymes. Partially purified preparations of rabbit antisera against each of the isozymes were prepared as described[17]; control serum was obtained prior to injection of extracts. Each sample contained 0.2 unit of pyrophosphatase activity and the specified amount of antisera protein, diluted to 0.3 ml with 0.85% (w/v) NaCl solution. After 5 min in ice, 1 ml of standard pH 9.0 assay mix, previously warmed to 30 °C, was added to initiate the reaction. ●—●, inhibition of Isozyme 2 by control serum; ▲—▲, antiserum to Isozyme 2; and ■—■, antiserum to Isozyme 1. Dotted lines are inhibition of Isozyme 1 by same antisera (same symbols).

Mixtures of the isozymes subjected to gel filtration on Sephadex G-150 with a series of molecular weight markers gave a single symmetrical peak of alkaline and acid (see later) inorganic pyrophosphatase activity; no resolution of the isozymes was observed. The observed apparent molecular weight (Fig. 5) was 32 000 + 4000. We

have previously reported a similar molecular weight, approximately 38 000, for this enzyme as extracted from a different maize hybrid[1]. It is of some interest to note that these molecular weights are approximately one-half that of crystalline yeast inorganic pyrophosphatase, 71 000 (ref. 18). The latter enzyme contains two apparently identical subunits[18]; it is conceivable that the maize leaf isozymes correspond to a single subunit of the yeast enzyme. Resolution of such questions awaits preparation of the maize leaf enzyme in homogeneous form. Other phosphohydrolase activities in the same maize leaf extracts yielded the following apparent molecular weights: acid phosphatase (EC 3.1.3.2), 90 000; and acid phosphodiesterase (EC 3.1.4.1), 170 000.

These results suggest that Isozymes 1 and 2 are of similar size but have significant structural differences as evidenced by their dissimilar thermal stabilities and their differences in susceptibility to inhibition by antisera.

*pH optima and metal ion specificity*

It has previously been reported from this laboratory[1] and elsewhere[3–5] that the optimum pH of this leaf inorganic pyrophosphatase ranges from pH 8 to 9,

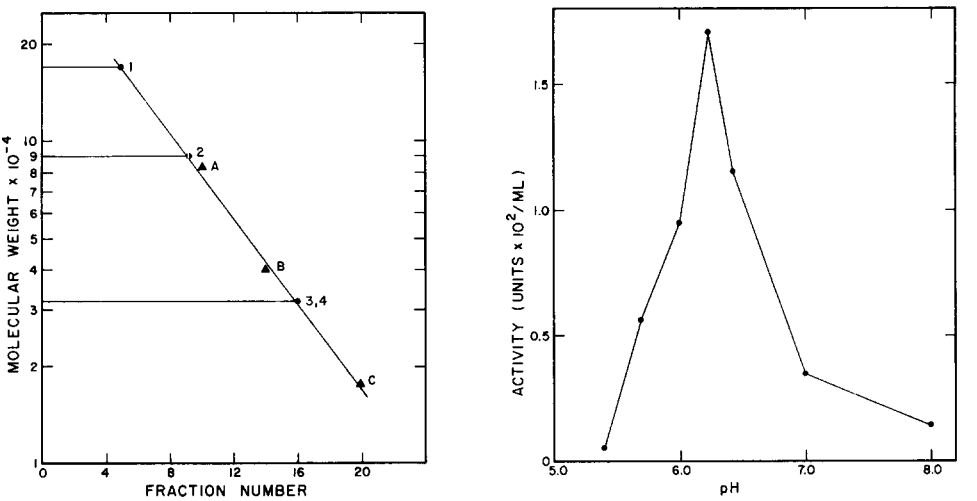


Fig. 5. Estimation of molecular weight by gel filtration. Marker enzymes were added to extract (1.0 ml) from 0.6 g fresh wt of light-grown maize leaves prepared in 0.1 M Tris–HCl, pH 8.0, containing 0.2 M KCl and 1 mM $Na_4P_2O_7$, and the mixture was applied to a 3 cm × 30 cm column of Sephadex G-150 (Pharmacia Fine Chemicals) which had been equilibrated with the above buffer. Fractions of 2.3 ml were collected every 10 min and assayed within a few hours. Marker enzymes were commercial preparations: (A) horse liver alcohol dehydrogenase, mol. wt 83 000 (ref. 19), assayed according to Dalziel[20]; (B) horseradish peroxidase, mol. wt 40 000 (ref. 21), assayed with guaiacol[22], and sperm whale myoglobin, mol. wt 17 800 (ref. 23), detected by its absorbance at 407 nm. Acid phosphatase (data point No. 2) was assayed with *p*-nitrophenyl phosphate as previously described[2]; acid phosphodiesterase (data point No. 1) was assayed similarly using bis (*p*-nitrophenyl phosphate) obtained from Aldrich Chemical Co. as substrate. Alkaline and acid pyrophosphatase (data points No. 3 and 4) assays were described in Materials and Methods.

Fig. 6. pH *vs* $Zn^{2+}$-activated pyrophosphatase activity. In order to avoid precipitation of zinc pyrophosphate the $^{32}P$ assay[1] was employed so that low concentrations of pyrophosphate could be employed. Conditions were $2.5 \cdot 10^{-5}$ M $Na_4P_2O_5$ and $1 \cdot 10^{-4}$ M $ZnCl_2$ in 0.05 M sodium cacodylate at the desired pH.

depending upon the concentration of $MgCl_2$ utilized to activate the reaction. These same studies indicated a high degree of specificity for $Mg^{2+}$ as the activating ion; no other divalent cation gave more than 10% of the activity obtained with $Mg^{2+}$. We have now observed that these same extracts contain potent acid pyrophosphatase activity which utilizes $Zn^{2+}$ as the activating ion. This activity has a sharp pH optimum at pH 6.2 (Fig. 6) and is relatively specific for $Zn^{2+}$ (Table I). $Co^{2+}$ and $Mg^{2+}$ partially activate the enzyme under these conditions; other metal ions tested either have no effect or somewhat inhibit the metal ion-independent acid pyrophosphatase activity. This metal ion requirement at pH 6.2 is much less specific for $Zn^{2+}$ than is the requirement for $Mg^{2+}$ at alkaline pH[1].

TABLE I

METAL ION SPECIFICITY OF ACID PYROPHOSPHATASE ACTIVITY

Assay conditions: 2 mM metal ion in 0.1 M ammonium cacodylate, pH 6.2, 1 mM $PP_i$.

| *Metal ion* | *Relative activity (%)* |
|---|---|
| $Zn^{2+}$ | 100 |
| $Co^{2+}$ | 32 |
| $Mg^{2+}$ | 15 |
| $Ni^{2+}$ | 8 |
| None, $Mn^{2+}$, $Fe^{2+}$ | 7 |
| $Ca^{2+}$, $Cd^{2+}$ | 5 |
| $Cu^{2+}$ | 3 |

Comparison of the acid and alkaline pyrophosphatase activities under standard, near-optimal conditions (acid pyrophosphatase: pH 6.2, 2 mM $Zn^{2+}$, 1 mM $PP_i$; alkaline pyrophosphatase: pH 9.0, 10 mM $MgCl_2$, 1 mM $PP_i$) gave a ratio of alkaline/acid activity of 1.5–2.0. A similar $Zn^{2+}$-activated acid pyrophosphatase activity was found in other plants, in amounts approximately equivalent to the alkaline $Mg^{2+}$ activated activity. In spinach, for example, we confirmed the presence of a highly active alkaline $Mg^{2+}$-activated pyrophosphatase[6], and found that the $Zn^{2+}$-activated acid pyrophosphatase activity is even higher so that the ratio of alkaline/acid activity is less than 1.

The question of whether the alkaline and acid activities are due to the same enzyme protein has been examined in several different ways. All attempts to separate the activities by chromatography, gel filtration (see Fig. 5), and isoelectric focusing have been uniformly unsuccessful. In all cases the activities exhibit identical elution patterns. A variety of treatments with antibiotics such as cycloheximide or chloramphenicol always resulted in a parallel change in both activities. The ratio of alkaline/acid activity was examined in the previously mentioned series of maize mutants and found to be rather constant (1.95, with a standard deviation of 0.366).

Activity determinations after analytical polyacryamide gel electrophoresis to separate the isozymes were consistent with both isozymes bearing both alkaline and acid pyrophosphatase activities, although these experiments are somewhat ambiguous because the separation of the isozymes by gel electrophoresis was run at alkaline pH. Incubation of the gels in the pH 6.2 assay with $Zn^{2+}$ resulted in development of the same pattern of the two isozymes as is seen by using the alkaline assay, but the

actual pH in the gel during the assay was probably somewhere between pH 6.2 and 9.0. Pre-rinsing of the gels in pH 6.2 buffer before assaying at pH 6.2 with $Zn^{2+}$ caused the resulting bands of activity to be faint and less distinct, although the resulting pattern was consistent with acid pyrophosphatase activity in both isozymes.

All of the data presently available suggest that the $Mg^{2+}$-activated activity at pH 8–9 and the $Zn^{2+}$-activated activity at pH 6.2 are manifestations of the same enzyme activity which is present in both isozymes. Thus, no functional difference has been observed in the two forms of the enzyme. It has been noted, however, that in some cases the activities are not equally stable; fresh extracts which exhibited normal amounts of both activities are found to lose the acid pyrophosphatase activity more readily on storage or manipulation.

*$K_m$ of acid pyrophosphatase*

The apparent $K_m$ for $PP_i$ for the $Zn^{2+}$-activated pyrophosphatase (present in the unpurified extract as a mixture of Isozymes 1 and 2) was determined at pH 6.2 using a large constant excess of $Zn^{2+}$ ($1.25 \cdot 10^{-4}$ M) and radioactive $^{32}PP_i$ ($2.1 \cdot 10^{-6}$ M to $5.2 \cdot 10^{-5}$ M) as previously described[1]. The apparent $K_m$ for $PP_i$ as obtained from linear plots of the data (not shown) was $0.6 \cdot 10^{-6}$ M, compared with a value of $5.6 \cdot 10^{-6}$ M found for the $Mg^{2+}$-activated alkaline pyrophosphatase[1]. These low $K_m$ values along with the relatively large amounts of the enzyme which are present[1,12], suggest that $PP_i$ is rapidly and efficiently broken down in the leaf even at extremely low concentrations.

*Responses to substrate analogs*

Two different analogs of inorganic pyrophosphate in which the bridge O atom is replaced, methylene diphosphonate and imidodiphosphate, were tested for their effect on this enzyme. At pH 9.0 with 2 mM $Mg^{2+}$ 1 mM imidodiphosphate was hydrolyzed at a rate no more than 1% of the corresponding rate with $PP_i$; the stable P–C bonds of methylene diphosphate disallow it as a substrate. Likewise, neither analog (1 mM) gave any detectable inhibition of $PP_i$ hydrolysis (1 mM) at pH 9.0 with 3.0 mM $Mg^{2+}$. Inhibition was observed only when analog concentrations were sufficiently high to compete with $PP_i$ for $Mg^{2+}$. In contrast yeast inorganic pyrophosphatase has recently been observed to be highly susceptible to competitive inhibition by imidodiphosphate but not methylene diphosphate[26]. The substrate and inhibitor specificities appear to be rather stringent for this maize leaf enzyme.

*Possible regulation by metabolites of the $C_4$ pathway*

It has been suggested[1,2] that this leaf pyrophosphatase has a primary function in driving the synthesis of phosphoenolpyruvate in those plants which fix $CO_2$ by the $C_4$ pathway[12]. We have examined several metabolites of this pathway for their possible regulatory effect upon maize leaf pyrophosphatase at metabolite concentrations from $10^{-6}$ to $10^{-2}$ M. In the pH 9.0, $Mg^{2+}$-activated assay two substrate concentrations (0.5 mM pyrophosphate, 5 mM $MgCl_2$; and 1 mM pyrophosphate, 10 mM $MgCl_2$) were employed; assays at pH 6.2 were carried out with 2 mM $Zn^{2+}$ and 1 mM $PP_i$. No detectable inhibition (other than competition for $Zn^{2+}$ at the highest metabolite concentrations) was observed at either pH by pyruvate, malate, oxaloacetate, ATP, ADP, AMP, 2-phosphoglycerate, 3-phosphoglycerate, or phosphoglycolate; 40–80%

inhibition was observed at $10^{-3}$–$10^{-2}$ M phosphoenolpyruvate at pH 9.0 only. Although similar inhibition by these levels of phosphoenolpyruvate has been observed for the enzyme immediately preceding inorganic pyrophosphatase in the $C_4$ pathway[24] these concentrations are probably much higher than physiological[25]; thus there is no evidence that the activity of leaf pyrophosphatase is regulated by metabolites of the $C_4$ pathway. A similar conclusion has been reached concerning the analogous enzyme from spinach chloroplasts[6].

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