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ALKALINE INORGANIC PYROPHOSPHATASE OF MAIZE LEAVES

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SUMMARY

1. Leaves of an albino mutant and of normal green seedlings of maize have high levels of readily extractable alkaline inorganic pyrophosphatase (EC 3.6.1.1) when grown under strong illumination. About one-tenth as much activity is found in both types of plants when grown in the absence of light. A brief illumination of dark-grown seedlings induces a large increase in readily extractable pyrophosphatase activity in the subsequent dark period.

2. In light-grown maize the enzyme is localized in the chloroplasts but is readily washed out by aqueous solvents. The enzyme is highly specific for Mg^{2+} and PP_1, and has optimal activity between pH 8 and 9. Leaf extracts of sorghum have similar high activity; many other plants, and other maize tissues have much less activity.

INTRODUCTION

We have examined the tissue of several higher plants as sources of enzymes catalyzing the hydrolysis of simple phosphoanhydrides. Preliminary experiments demonstrated the presence of potent inorganic pyrophosphatase activity (EC 3.6.1.1) in leaf extracts of maize (*Zea mays*) and related plants. Reported here are some properties of the maize enzyme, the effect of light on the level of extractable enzyme, its intracellular location, and the occurrence of this activity in other species. All of these observations are consistent with an indirect yet specific role of this enzyme in the novel C_4 dicarboxylic acid pathway of photosynthetic CO_2 fixation[1]. A preliminary account of this work has been presented[2].

METHODS

Albino maize mutant seeds (E-M strain 1) were obtained from General Biological Supply House, Chicago, Ill.; all other seeds were obtained from the Seed Laboratory of the Indiana State Seed Commissioner.

Seeds were soaked several hours in distilled water, planted in potting soil, and grown at room temperature in a greenhouse (natural illumination only) or laboratory (artificial illumination only). Where utilized, 240 ft candles of artificial illumination were provided by a Champion 150-W incandescent reflector lamp 1.5 m above the plants. No special precautions were taken to exclude light during preparations of extracts from etiolated tissue because of the observed slow response to light.

Unless otherwise specified, 2–3-week-old plants were utilized. Control experiments indicated that the pyrophosphatase activity of maize leaf tissue varies only slightly with age. Fresh tissue was homogenized by grinding with an equal weight of

Superbrite glass beads (3 M Company, St. Paul, Minnesota) in a chilled mortar, using 10 or 20 ml of cold 50 mM Tris acetate buffer (pH 7.5) per g of tissue. The supernatant fraction obtained after centrifugation for 10 min at 5000 × *g* was assayed immediately, although little activity was lost in samples stored overnight at 3°.

Hydrolysis of PP_i was routinely determined in a 1-ml reaction mixture containing 1 mM tetrasodium pyrophosphate, 100 mM Tris acetate buffer (pH 9.0), 10 mM $MgCl_2$ and aliquots of enzyme suitably diluted so that no more than 0.3 μmole of PP_i was hydrolyzed. After incubation for 10 min at 30°, the mixture was analyzed for P_i by a scaled-up version of JOSSE's modified Fiske and SubbaRow procedure[3], except that the reaction was stopped by addition of the acid molybdate mix rather than chilling to 0°. A unit of enzyme activity is that amount which will hydrolyze 1 μmole of PP_i per min in this assay. Unless otherwise specified, the source of enzyme was extract of maize leaves (Indiana Hybrid 682) grown under continuous artificial illumination.

Protein concentration was determined according to LOWRY *et al.*[4], using crystalline bovine serum albumin as standard. Chlorophyll was determined by the method of ARNON[5].

RESULTS

Catalytic characteristics

The crude maize-leaf enzyme has an absolute requirement for Mg^{2+}. At the same concentration and pH, all other metals tested are less than 1% as effective as Mg^{2+} (Table I). 1 mM Ca^{2+} and F^- strongly inhibit the Mg^{2+}-activated reaction. Large excesses of Mg^{2+} are only slightly inhibitory and only above pH 8, as seen in Fig. 1.

TABLE I

METAL ION SPECIFICITY

Hydrolysis of PP_i was measured as described in METHODS, with suitable substitution for $MgCl_2$.

	Relative rate
Mg^{2+}	1000
Mn^{2+}	6
Zn^{2+}	5*
Fe^{2+}	5*
Co^{2+}	4
K^+	4
Ca^{2+}	0*
Cd^{2+}	0*
No addition	0

* Incipient precipitation during the assay.

The apparent sigmoid-type curves probably reflect the relief of inhibition of the enzyme by uncomplexed PP_i as the concentration of Mg^{2+} is increased, although allosteric activation of the enzyme by Mg^{2+} has been postulated for pyrophosphatase of mouse liver cytoplasm[6]. The rate of PP_i hydrolysis as a function of PP_i concentration, measured with $5 \cdot 10^{-7}$–10^{-4} M $^{32}PP_i$ in the presence of 10^{-2} M $MgCl_2$, yielded an apparent K_m for PP_i of $5.6 \cdot 10^{-6}$ M (Fig. 2).

The maize enzyme has maximal activity between pH 8 and 9 (Fig. 3), and the optimum shifts slightly toward lower pH values with larger excesses of Mg^{2+}, as has been observed with yeast inorganic pyrophosphatase[7].

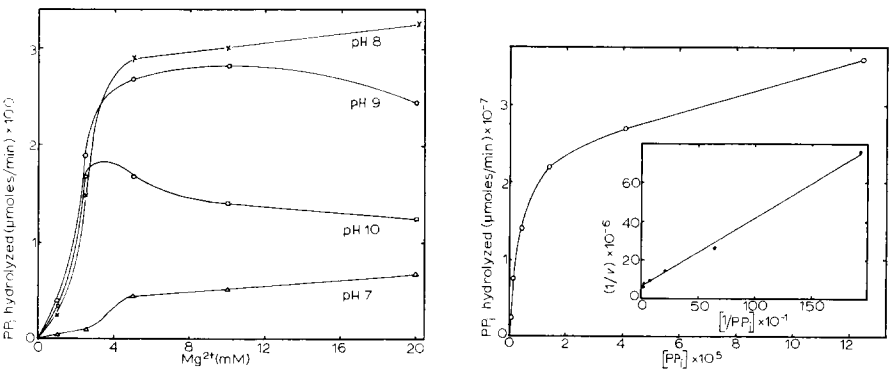

Fig. 1. Effect of $MgCl_2$ concentration on PP_i hydrolysis at different pH values. Standard pyrophosphatase assay except for adjustment of $MgCl_2$ and H^+ concentration.

Fig. 2. Effect of PP_i concentration on hydrolysis rate. Standard conditions except for adjustment of PP_i concentration. $^{32}PP_i$ (20000 counts/min per mμmole in the lowest concentration used) was obtained from New England Nuclear Corporation. Conversion to P_i was measured by appearance of ^{32}P in the upper layer on distribution between isobutanol–benzene (1:1, v/v) and 1.5 % ammonium molybdate in 0.25 M H_2SO_4 (ref. 9). Aliquots were dried on planchets and ^{32}P determined in a Nuclear-Chicago gas flow end-window counter.

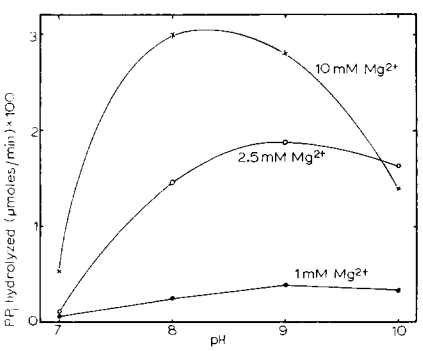

Fig. 3. Effect of pH on PP_i hydrolysis with various concentrations of $MgCl_2$. Standard pyrophosphatase assay except for adjustment of H^+ and $MgCl_2$ concentration.

The relative rates of hydrolysis of a series of potential substrates by the crude maize-leaf extract are presented in Table II. Under conditions which are optimal for pyrophosphatase but may not be optimal for the other substrates, fructose 1,6-diphosphate is hydrolyzed at about 2 % the rate of PP_i cleavage, and all other phosphoanhydrides and phosphomonoesters tested are hydrolyzed more slowly, if at all. Other studies indicated that adenine nucleotides, cyclic hexametaphosphate, citrate, malonate, and bicarbonate either do not inhibit the hydrolysis of PP_i or inhibit only to the extent that they compete for Mg^{2+}.

Gel filtration on Sephadex G-100 along with *E. coli* alkaline phosphatase, bovine serum albumin, horseradish peroxidase, and bovine heart cytochrome *c* as molecular

TABLE II

PHOSPHATASE ACTIVITY OF MAIZE-LEAF EXTRACT

Standard assay conditions were 10 mM $MgCl_2$, 100 mM Tris buffer (pH 9), 30°. Hydrolysis of PP_i, cyclic hexametaphosphate (Fisher Scientific Company, Fair Lawn, New Jersey), ATP, glucose 6-phosphate, and fructose 1,6-diphosphate was measured by P_i appearance as described in METHODS. Hydrolysis of *p*-nitrophenyl phosphate was followed spectrophotometrically at 400 mμ. Phthaloyl monophosphate was prepared according to HIGUCHI, FLYNN AND SHAH[8], and measured spectrophotometrically at 290 mμ.

Substrate	*Conc. (mM)*	*Rate (relative)*
PP_i	1.0	1000
Fructose 1,6-diphosphate	3.2	21
Cyclic hexametaphosphate	1.0	<6
ATP	1.0	4.3
Glucose 6-phosphate	1.85	<3.6
Phthaloyl monophosphate	0.2	<1.2
p-Nitrophenyl phosphate	0.05	0.3
PP_i (pH 6)	1.0	114
PP_i (pH 6, no Mg^{2+})	1.0	36

weight markers indicates an apparent molecular weight[10] of approx. 38 000, about one-half that of the corresponding yeast enzyme[11].

Response to light

Table III documents the correlation of extractable pyrophosphatase activity of a normal photosynthetically competent maize hybrid and an albino mutant with the level of illumination of the plants during growth. The activity of leaf extracts from

TABLE III

EFFECT OF DIFFERENT LEVELS OF ILLUMINATION ON PYROPHOSPHATASE ACTIVITY OF MAIZE LEAVES

	Units/g tissue	
	Normal green seedlings	*Albino mutant*
Etiolated	6.3	2.7
Greenhouse (no artificial light)	17.7	10.0
Continuous artificial illumination	42.0*	34.2

* 1.42 units/mg extracted protein, 102 units/mg extracted chlorophyll.

plants grown under continuous illumination is several-fold higher than that of etiolated plants, and greenhouse plants have intermediate levels. The low levels of activity found in the stem and root (Table IV) were only marginally increased by growth in light, and the activity of light-grown leaves did not decline in the dark over a period of several days.

The nonphotosynthetic albino mutant is similarly sensitive to light, reaching levels of pyrophosphatase activity near those of the normal hybrid, indicating that light may serve solely as an initiator of synthesis or activation of the enzyme. This

TABLE IV

DISTRIBUTION OF ALKALINE PYROPHOSPHATASE IN GREENHOUSE-GROWN PLANTS

Activity is expressed as μmoles PP_i hydrolyzed per min per g tissue extracted.

Plant	*Activity*
Sunflower (*Helianthus annuus*)	1.1
Soybean (*Glycine max*, var. Amsoy)	1.6
Coleus (*Coleus blumei*)	1.9
Oats (*Avena sativa*)	3.0
Ryegrass (*Lolium italicum*)	3.1
Watermelon (*Citrullus vulgaris*)	3.1
Squash (*Cucurbita moschata*)	3.3
Wheat (*Triticum sativum*)	5.8
Rye (*Secale cereale*)	6.5
Barley (*Hordeum vulgare*)	7.7
Sorghum (*Sorghum vulgare*, var. Atlas Sorgo)	19.3
Maize (*Zea mays*, Indiana Hybrid 682)	23.2
Maize, stem	1.05
Maize, root	0.25
Maize, mature seed	0.0

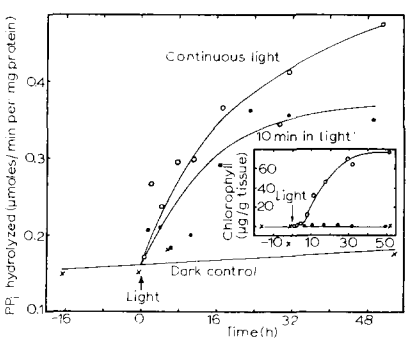

Fig. 4. Effect of different lengths of illumination on the pyrophosphatase activity of etiolated maize plants. Etiolated maize seedlings were 6 days old at the beginning of the experiment. All the tissue above the node from 5 plants in each group was homogenized, extracted, and assayed in triplicate for each data point. ×, dark controls; ○ etiolated seedlings exposed to light from 0 h onward; ●, etiolated seedlings exposed to light for 10 min beginning at 0 h. Chlorophyll assays on the same aqueous extracts.

possibility is enhanced by the experiment depicted in Fig. 4. Etiolated maize seedlings were briefly illuminated and the alteration of pyrophosphatase activity in the subsequent dark period was compared to that of etiolated plants brought into light at the same time and left there. A 10-min exposure to light resulted in an increase in activity similar in rate and extent to that caused by continuous illumination. The activity of dark controls did not increase significantly, and the level of extractable protein was not affected by illumination. In agreement with previous reports[12,13], no detectable chlorophyll was formed as a result of the brief exposure to light (Fig. 4, insert), showing that photosynthetic reactions are not necessary to 'trigger' the increase in enzyme activity. The data reported in Fig. 4 were obtained by extracting all tissue above the node, including considerable relatively inactive stem tissue (Table IV), so

that the observed increases in activity are less striking than the differences reported in Table III, in which only leaf tissue was analyzed.

Distribution. The inorganic pyrophosphatase activity readily extracted from the leaves of a variety of species is shown in Table IV. Most dicotyledonous plants and conifers have 1–5 units/g of tissue. Some of these extracts contain a dialyzable component, not destroyed by ashing, which strongly inhibits the maize enzyme. The activity of these extracts was not increased by dialysis, thus excluding the possibility that the low observed activity was due to the presence of the inhibitor.

Cereal grains and grasses have somewhat more activity, 3–8 units/g tissue. A previous report[14] indicated relatively large amounts of alkaline inorganic pyrophosphatase activity in "grass". Maize and sorghum are especially active, having 2–3-fold higher activity than any other plants tested. All varieties of maize tested, including several hybrids, purple Indian corn, and an albino mutant, are similarly potent. Extracts of maize root, stem, and seed have less than 5 % of the activity of leaf extracts.

Subcellular localization. The location in the leaf of the major portion of the pyrophosphatase activity, the response of etiolated leaves to illumination, and previous reports of the intracellular location of other specific phosphatases[15,16], suggested the possibility that the enzyme is located in chloroplasts. Aqueous preparations of chloroplasts contained less than 5 % of the total pyrophosphatase activity, even under special conditions specifically adapted to isolation of maize chloroplasts with optimal photophosphorylation rates[17].

Nonaqueous preparations have been employed to demonstrate the association with chloroplasts of several enzymes of the Calvin cycle which are washed out in aqueous preparations[15]. Our nonaqueous preparations[18] of maize chloroplasts contained a large fraction of the total pyrophosphatase activity of the leaf. Representative data on the activity of several enzymes assayed in the nonaqueous chloroplasts are shown in Table V. When expressed on the basis of chlorophyll content, pyrophosphatase activity of the nonaqueous chloroplasts is virtually identical to that of an aqueous homogenate of the same lyophilized leaf tissue from which the nonaqueous chloro-

TABLE V

INTRACELLULAR LOCATION OF PYROPHOSPHATASE

Maize-leaf chloroplasts (density $\leqslant$ 1.32 g/ml) prepared as described by STOCKING[18] were compared to aqueous homogenates of the same lyophilized maize leaves. Pyrophosphatase activity and chlorophyll were determined as described in METHODS, and the fructose diphosphatase assay was described in Table II. NADPH diaphorase was measured by the procedure of JAGENDORF[20]. Acid phosphatase was measured at pH 5.6 with *p*-nitrophenol as substrate[1].

Enzyme	*Activity (μmoles/min per mg chlorophyll)*		*Activity in chloroplasts* (%)
	Chloroplast	*Leaf*	
Pyrophosphatase	16.1	16.3	99
Diaphorase (NADPH)	2.1	3.08	68
Fructose diphosphatase	0.247	0.632	39
Acid phosphatase (arbitrary units)	1.25	3.46	36

plasts were prepared. NADPH diaphorase, previously shown to be located almost exclusively in the chloroplast fraction of spinach leaves[19], is slightly lower in this preparation than in the aqueous homogenate, but in other preparations paralleled the pyrophosphatase activity even more closely. In contrast, a relatively low amount of acid phosphatase, a cytoplasmic enzyme[1], is recovered in the chloroplast preparation. Alkaline pyrophosphatase of maize fractionates with chlorophyll and therefore appears to be associated with chloroplasts in the intact leaf, but is readily washed out in aqueous media. Alkaline fructose 1,6-diphosphatase of pea leaf and EUGLENA is localized in chloroplasts[15], but most of this activity in maize leaves occurs in the cytoplasm.

DISCUSSION

The chemical and physical properties of the leaf enzyme are similar in many respects to inorganic pyrophosphatases obtained from yeast[7], *E. coli*[3], mouse liver cytoplasm[6], and human erythrocytes[21]. This enzyme appears to be a major phosphohydrolase activity of maize leaf extracts. The high specific activity, 1–2 μmoles/min per mg protein, and the narrow specificity for substrate and metal ion are noteworthy for a crude plant extract.

A recently proposed novel pathway of photosynthetic CO_2 fixation[1] involves phosphopyruvate synthetase:

$$\text{Pyruvate} + \text{ATP} + P_i \rightleftarrows \text{phosphopyruvate} + \text{AMP} + PP_i \qquad (1)$$

and phosphopyruvate carboxylase:

$$\text{Phosphopyruvate} + CO_2 \longrightarrow \text{oxaloacetate} + P_i \qquad (2)$$

The much higher pyrophosphatase activity of maize and sorghum than other plants, as reported here, closely parallels the distribution of the above enzymes[1,22]. The rate of PP_i hydrolysis by maize is greater than maximum rates of photosynthesis, and pyrophosphatase activity is a persistent impurity in preparations of phosphopyruvate synthetase[22]. These considerations, along with the intracellular localization in maize leaf chloroplasts of both phosphopyruvate synthetase[23] and pyrophosphatase, suggest that the primary role of the pyrophosphatase is to drive the phosphopyruvate synthetase reaction toward phosphopyruvate formation by removal of the product PP_i, as proposed by HATCH AND SLACK[22].

This proposed role is consistent with the far greater pyrophosphatase activity of the leaf than in the other tissues of the plant, in which the observed low levels of activity are apparently sufficient to meet the needs for driving ubiquitous activation reactions. Thus, the inorganic pyrophosphatase of leaves of maize and sorghum may contribute to the high efficiency of photosynthetic CO_2 fixation by these plants and may be more exclusively involved in a particular metabolic process than any inorganic pyrophosphatase previously described.

It is of some interest to ascertain whether etiolated albino maize mutants (green:white:yellow; 9:4:3) respond to brief illumination in the same manner as normal, potentially green, plants. No suitable method was found for selecting albinos out of groups of etiolated seedlings containing both albino and normal plants, but analogous experiments with the bulk mixture of albino and normal seedlings grown in the dark showed virtually no differences in the levels of pyrophosphatase activity

between continuous illumination and a brief illumination followed by darkness. Thus the light sensitivity of the albino mutants appears to be qualitatively similar to that of normal plants, indicating a rather simple system of chromophores for activating the photoresponse. KLEIN AND FILNER[24] have presented evidence that synthesis of several enzymes in etiolated bean seedlings is induced by a brief exposure to red light. Induction is reversed by subsequent exposure to far-red light, indicating that phytochrome is probably the chromophore responsible for the induction. Our data show that under conditions where photosynthesis is not possible (in the dark after a brief light exposure) or severely impaired (in the albino mutant), synthesis or activation of maize-leaf pyrophosphatase occurs in a manner which is qualitatively and quantitatively similar to that which occurs in conditions favoring photosynthesis as a source of energy. The similarities between the maize and bean systems suggest phytochrome as the chromophore responsible for light induction of maize-leaf pyrophosphatase.

A phosphopyruvate synthetase in leaves of *Amaranthus palmeri*, which also fixes CO_2 by the C_4 dicarboxylic acid pathway[23], is light sensitive, showing large increases and decreases in activity only a few minutes after exposure to light and removal of light, respectively[23]. In contrast, our studies of maize leaves indicate that the light-induced increase in the apparently functionally related enzyme, inorganic pyrophosphatase, is irreversible and relatively slow, presumably reflecting a less direct role in photosynthetic CO_2 fixation.

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