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CHLOROPLAST INORGANIC PYROPHOSPHATASE

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SUMMARY

An alkaline, Mg^{2+}-dependent inorganic pyrophosphatase has been isolated from previously isolated spinach chloroplast. The activity of the enzyme was increased 100-fold, with a 42 % yield, upon purification from the total soluble chloroplast enzymes. The pH optimum for the enzyme shifts from 9.0 at 5 mM Mg^{2+} to 7.0 at 40 mM Mg^{2+}. The substrate for the reaction appears to be magnesium pyrophosphate, and anionic pyrophosphate is an effective inhibitor. There seems to be also an activating effect of Mg^{2+} on the enzyme at pH 7. No other cation substitutes for Mg^{2+} in activating the hydrolysis of pyrophosphate. Among anions tested, only F^- caused severe inhibition. The enzyme is inactive towards fructose 1,6-diphosphate, thiamine pyrophosphate, ATP, and ADP. The possibility that this enzyme is subject to metabolic regulation is discussed in relation to an indicated role of pyrophosphate in the regulation of photosynthetic carbon reduction.

INTRODUCTION

Enzymes which hydrolyze inorganic pyrophosphate (PP_i) to orthophosphate have been found to be widely distributed in animal, plant, and bacterial systems[1–18]. However, the enzyme, obtained from previously isolated chloroplasts, is required for studies of the possible role of this reaction in photosynthesis. For example, if chloroplast pyrophosphatase activity is subject to metabolic regulation, this control might be effected by a different means in the photosynthetic system than in other metabolic systems. This is the case for fructose diphosphatase[19,20]. We report here the finding of alkaline pyrophosphatase activity in previously isolated spinach chloroplasts, methods of purification and characterization of the enzyme, and the dependence of its activity on Mg^{2+} concentration and pH. SIMMONS AND BUTLER[18] found that maize leaves contain alkaline inorganic pyrophosphatase activity, with a specific requirement for Mg^{2+} and a pH optimum between 8 and 9.

MATERIALS AND METHODS

Chemicals

Tetrasodium pyrophosphate, Allied Chemical; AMP, ADP, ATP, TPP, *p*-nitrophenylphosphate, CalBiochem, grade A; fructose 1,6-diphosphate, sodium salt, Sigma Chemical Co. All inorganic salts were analytical reagents.

Enzyme assay

The enzymatic hydrolysis of inorganic pyrophosphate was determined in a 1-ml reaction mixture containing 2 mM tetrasodium pyrophosphate, 50 mM Tris buffer (pH 7–9), 5–40 mM $MgCl_2$, 5 mM EDTA, and the required amount of the enzyme for obtaining a detectable amount of orthophosphate. The reaction was stopped by the introduction of 0.1 ml of 50 % trichloroacetic acid in aqueous solution and the protein was spun down. Aliquots of the supernatant were used for analyzing the released orthophosphate by the method of FISKE AND SUBBAROW[21] (ref. 22).

Protein determination

In the process of enzyme purification, in order to determine the specific activity of the enzyme, protein determinations were carried out using ultraviolet absorption at 280 nm for preliminary estimation. The LOWRY[23] method of protein determination was used for more precise results. Bovine serum albumin was used as the standard in each of the two methods.

Isolation of the enzyme inorganic pyrophosphatase

Spinach chloroplast inorganic pyrophosphatase was isolated using the following method:

Source of the enzyme

Field-grown spinach leaves were harvested and immediately stored over ice in polyethylene bags in large ice chests. The leaves were deribbed and washed with ice-cold water and were dried between two sponges. The deribbed leaves were then chopped and divided into 50-g batches.

Isolation of chloroplasts

Each 50-g batch was homogenized for 8 sec in a Waring blendor with 200 ml of solution A (ref. 24) (containing 0.33 M sorbitol, 0.002 M $NaNO_3$, 0.002 M EDTA (dipotassium salt), 0.002 M sodium isoascorbate, 0.001 M $MnCl_2$, 0.001 M $MgCl_2$, 0.0005 M KH_2PO_4, 0.05 M 2-*N*-morpholinoethane sulfonic acid adjusted with NaOH to pH 6.1, and 0.02 M NaCl). The blendorate was then forced through six layers of cloth to strain out fibrous material.

The homogenate was centrifuged at 2000 $\times$ *g* for 3 min. The supernatant was decanted and each pellet was suspended in 10 ml of "basic buffer" (0.05 M Tris buffer (pH 7.4) – 0.002 M dithiothreitol – 0.0002 M EDTA – 0.001 M $MgCl_2$).

Sonication

The chloroplast suspension was sonicated for 30 sec in batches of 50 ml, using the Biosonik (Model BPI, Bronwill Scientific Co., Rochester, N.Y.) at 0°.

The sonicated suspension was centrifuged at 36000 $\times$ *g* for 2 h and the suspension was saved as the crude enzyme preparation (1).

Acetone fractionation

Acetone was added to the enzyme to a concentration of 30 %. The acetone had been precooled in the freezer at —14° and was added to the crude enzyme solution slowly while stirring at 4°. The enzyme in 30 % acetone was allowed to stand in the

cold room (4°) for 30 min and the mixture was centrifuged at 13200 × *g* for 4 min. The supernatant was collected and the acetone concentration in the supernatant was brought to 75 %. The enzyme in 75 % acetone was allowed to stand in the freezer at −14° for 1–2 h. A copious precipitate formed and settled toward the bottom of the container. The upper layer of 75 % acetone solution was decanted. The lower layer containing the precipitated enzyme and some other proteins was then centrifuged for 1 min at 5000 × *g* and the pellets were collected.

The greyish-white precipitate was dissolved in the smallest possible volume of basic buffer and was dialyzed against cold water (4°) for 4 h. Then it was dialyzed against basic buffer twice, for 8 h each time.

The dialyzed mixture was centrifuged in the Sorvall, at 36000 × *g* for 10 min, and the supernatant (II) was saved.

First DEAE-cellulose column

A DEAE-cellulose column was prepared and pre-equilibrated with 0.05 M Tris–HCl (pH 7.4). The supernatant was applied to the column and the column was washed with 0.05 M Tris–HCl buffer (pH 7.4) until no more protein was found in the eluate. Then the column was eluted with a buffer containing 0.15 M Tris–HCl and 0.28 M NaCl (pH 7.4)(III).

Second DEAE-cellulose column

The protein eluted from the first column (III) was diluted to bring the salt concentration down to 0.05 M and was applied to a DEAE column that had been equilibrated with 0.05 M Tris–HCl (pH 7.4). A gradient of 0.05 M NaCl and 0.05 M Tris buffer (pH 7.4) to 0.15 M Tris–HCl and 0.5 M NaCl (pH 7.4) was used for elution, and fractions that contained pyrophosphatase activity were pooled (IV).

The specific activity of the purified enzyme (IV) was increased 100-fold (see Table I) as compared with the total soluble chloroplast protein (I).

Stabilization of pyrophosphatase activity

Among the sulfhydryl reagents used, dithiothreotol had the greatest stabilizing effect on the enzyme. Glutathione at a comparable molarity (0.002 M) also had a good stabilizing effect. The enzyme is very stable in Tris–HCl buffer (pH 6–9.5). EDTA is needed at a concentration of 0.0002 M to stabilize pyrophosphatase activity. EDTA at the relatively high concentration of 0.002 M was tolerated by pyrophosphatase. The

TABLE I

PURIFICATION OF SPINACH CHLOROPLAST INORGANIC PYROPHOSPHATASE

A unit of enzyme activity is that amount which will hydrolyze 1 μmole of PP_i per min in the described assay.

Fraction	*Protein (mg/ml)*	*Specific activity (units/mg protein)*	*Yield (%)*
I Sonicate supernatant	10	12	100
II Acetone fraction	20	230	57
III First DEAE-cellulose column	10	520	49
IV Second DEAE-cellulose column	6	1230	42

high concentration of EDTA was essential as a bacteriostatic agent in the storage solution. Among all bactericidal and bacteriostatic agents tested, EDTA at 0.002 M was the most satisfactory. Pyrophosphatase activity remained unchanged for 2 months in 0.05 M Tris buffer (pH 6.2), 0.002 M EDTA, 0.002 M dithiothreotol at 0° C. However, pyrophosphatase activity was not decreased after heating for 20 min at 60°.

RESULTS

Effect of substrate concentration on the activity of pyrophosphatase at different Mg^{2+} concentrations and the corresponding pH optima

This experiment was designed to study the substrate effect at two different Mg^{2+} concentrations. These were Mg^{2+} concentrations of 5 mM at pH 9 and 40 mM at pH 7.

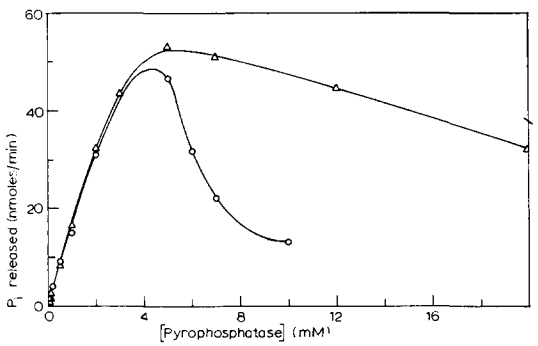


Fig. 1. Effect of pyrophosphate concentrations on pyrophosphatase activity at two levels of Mg^{2+} concentration: 5 mM (○——○) and 40 mM (△——△). Assays were carried out in 1 ml of assay mixture containing 40 ng of Fraction IV protein.

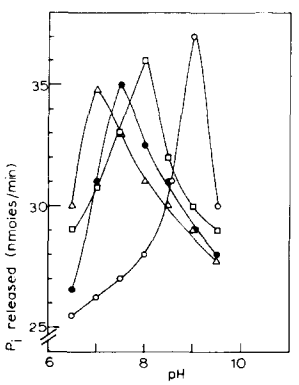
The enzyme concentration was constant and the only variable in this experiment was the concentration of the substrate PP_i. It was found that pyrophosphatase was inhibited by the substrate at higher concentrations (Fig. 1). At 5 mM Mg^{2+} concentration and pH 9, the optimum pyrophosphate concentration for maximum pyrophosphatase activity was 5 mM. At pH 7 and higher Mg^{2+} concentration, the optimum substrate concentration was also 5 mM; however, only slight inhibition of pyrophosphatase activity was observed at higher substrate concentrations. This observation could be taken to indicate that Mg^{2+} exerts an effect on the substrate. Magnesium pyrophosphate may be the specific substrate for the enzyme and anionic pyrophosphate itself an inhibitor. Thus the higher Mg^{2+} concentration would result in the availability of a higher concentration of the specific substrate magnesium pyrophosphate and therefore would counteract the effect of the competitive inhibitor anionic inorganic pyrophosphate.

Effect of Mg^{2+} on the pH optimum of pyrophosphatase

In studying the effect of Mg^{2+} on the pH optimum for inorganic pyrophosphatase activity, Mg^{2+} concentrations of 5 mM to 40 mM were used and the pH of the reaction mixture was varied from pH 7 to 9.5. Fig. 2 shows the shift of two pH units from an optimum of pH 9 at a Mg^{2+} concentration of 5 mM to an optimum of pH 7 at 40 mM Mg^{2+}. The effect of Mg^{2+} on pH optimum could be due to one or both of the following factors:

(1) The effect of Mg^{2+} is on the substrate. That is, the substrate for the enzyme is not free pyrophosphate anion, but magnesium pyrophosphate.

(2) Mg^{2+} exerts its effect directly on the enzyme. That is, Mg^{2+} alters the conformation or the ionic properties of the active site of the enzyme pyrophosphatase. The direction of the shift in pH optimum seems to favor the assumption that Mg^{2+} affects the enzyme as well as the substrate. One explanation could be that Mg^{2+} helps cover some negative charges near the active site at pH 7 which interfere with the accessibility of the active site to the substrate. If, at pH 9, the conformation of the enzyme has changed so that the negative charges are removed from the vicinity of the active site, Mg^{2+} would be needed only to make the specific substrate, magnesium pyrophosphate. Thus, these results suggest that Mg^{2+} exerts its effect on both substrate and enzyme.



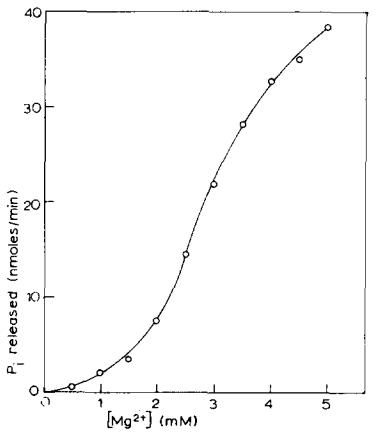


Fig. 2. Effect of Mg^{2+} concentration on pH optimum. Assays were carried out in 1 ml assay mixture containing 40 ng of Fraction IV protein. ○——○, 5 mM Mg^{2+}; □——□, 10 mM Mg^{2+}; ●——●, 20 mM Mg^{2+}; △——△, 40 mM Mg^{2+}.

Fig. 3. Effect of lower concentrations of Mg^{2+} on the activity of pyrophosphatase. Assays were carried out in 1-ml mixtures containing 40 ng of Fraction IV protein.

The effect of lower concentrations of Mg^{2+} on the pyrophosphatase activity was determined in an assay mixture containing 2 mM tetrasodium pyrophosphate, 50 mM Tris buffer (pH 9) in the absence of EDTA. However, 5 mM of EDTA was found to have no effect on the enzyme activity at all levels of Mg^{2+} concentration. The sigmoidal dependence of enzyme activity on low concentrations of Mg^{2+} (Fig. 3) provides evidence of an allosteric effect of Mg^{2+} on the activity of the enzyme[25]. Anionic pyrophosphate may have also contributed to the sigmoidal shape of the curve at low Mg^{2+} concentrations.

Effect of cations on pyrophosphatase activity

Among the 12 cations (Table II) tested, only Mg^{2+} markedly stimulated the activity of the enzyme pyrophosphatase. Au^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Ca^{2+} had no activating effect at low concentration and an inhibitory one at higher concentrations. No activities for hydrolysis of ATP, ADP, or TPP were induced by presence of Zn^{2+} or Mn^{2+} as was found for pyrophosphatase from yeast[26]. None of the cations tested could replace or match the activating effect of Mg^{2+} on pyrophosphate.

TABLE II

EFFECT OF CATIONS ON PYROPHOSPHATASE ACTIVITY

Cation	*nmoles of P_i released/ml* per min*			
	5 mM cation	*10 mM cation*	*20 mM cation*	*40 mM cation*
Mg^{2+}	34	32.5	30	26
Mn^{2+}	2.1	1.5	1.5	1.5
Zn^{2+}	2.75	2.0	1.0	1.0
Fe^{3+}	0.5	0.4	0.25	0.05
Co^{2+}	1.6	1.1	1.5	1.6
Ni^{2+}	1.25	1.0	1.25	1.0
Au^{2+}	0.53	0.5	1.0	1.0
Cd^{2+}	1.0	1.0	1.0	1.5
Ca^{2+}	1.5	1.0	0.75	1.25

*Containing 40 ng Fraction IV protein.

TABLE III

EFFECT OF ANIONS ON PYROPHOSPHATASE ACTIVITY

Anion	*nmoles of P_i released/ml* per min*			
	5 mM anion	*10 mM anion*	*20 mM anion*	*40 mM anion*
F^-	3.85	3.5	1.25	0.5
Cl^-	31.5	29.5	30.0	27.0
Br^-	31.5	28.0	27.0	30.0
I^-	31.5	28.0	27.0	29.5
SO_4^{2-}	26.5	25.0	27.25	23.0

*Containing 40 ng Fraction IV protein.

Effect of anions on the activity of pyrophosphatase

In testing for the effect of anions on the activity of pyrophosphatase, Mg^{2+} was always present at a concentration of 5 mM. The pH of the reaction medium (0.05 M Tris–HCl) was 9 and pyrophosphate was present in sufficient amounts (2 mM) to yield detectable quantities of P_i product. The anions F^-, Br^-, Cl^-, I^-, SO_4^{2-}, and HPO_4^{2-} were tested individually at a concentration of 5 mM. Only F^- was found to have an inhibitory effect on the enzyme in the presence of Mg^{2+} (Table III). The effect of F^- may be due to prevention of Mg^{2+} activation of the pyrophosphatase enzyme due to the low solubility product of MgF_2. The fluoride inhibition was striking.

Specificity of pyrophosphatase

In order to determine the specificity of pyrophosphatase, different potential substrates were incubated with the enzyme at pH 9 and in the presence of 5 mM Mg^{2+}. The reaction was run at room temperature for 20 min. Pyrophosphatase was found to be very specific for inorganic pyrophosphate. When tested at pH 9 and 5 mM Mg^{2+}, pyrophosphatase was inactive toward fructose 1,6-diphosphate, ATP, ADP, thiamine pyrophosphate, and *p*-nitrophenylphosphate.

Effect of inhibitors of CO_2 fixation on the activity of pyrophosphatase

In studying the effect of several inhibitors of CO_2 fixation on pyrophosphatase activity, optimum conditions for enzyme activity were employed (pH 9, Mg^{2+} concentration of 5 mM ,4 mM pyrophosphate) and the reaction was run at room temperature. Vitamin K_5, caprylic acid, and the natural spinach inhibitor (factor B)[31] were tested. Vitamin K_5, caprylic acid, and the spinach juice factor showed no inhibitory effect on the pyrophosphatase activity. AMP, ADP, and ATP in the presence of Mg^{2+} fail to exert any inhibitory effect on catalytic activity of the enzyme towards pyrophosphate in contrast to the enzyme from *Escherichia coli* (ref. 12).

DISCUSSION

The finding of Mg^{2+}-dependent, inorganic pyrophosphatase in previously isolated spinach chloroplasts supports the proposal that the level of PP_i inside the chloroplasts is controlled within the chloroplasts. The dependence of the pH optimum on Mg^{2+} level in the range of 5–40 mM shows some similarity to the behavior of fructose-1,6-diphosphatase[20] (EC 3.1.3.10) and of ribulose diphosphate carboxylase[27] (EC 4.1.1.39), both of which exhibit Mg^{2+}-dependent shifts in pH optima over about the same range of pH and Mg^{2+} concentration. The absolute change in activities with pH and Mg^{2+} concentration are different for each of the three enzymes.

It has been suggested that the mechanism of light–dark regulation of diphosphatase and carboxylase enzymes of the carbon reduction cycle might depend on changes in Mg^{2+} concentration and pH resulting from light-pumping of ions through the thylakoid membranes, leading to a higher pH and Mg^{2+} concentration in the stroma region of the chloroplasts in the light[28]. Light-induced pumping of these ions in chloroplasts has been reported[29], but unfortunately information about the possible changes induced in the stroma region of intact chloroplasts is not available. If the suggested mechanism is correct, and if pyrophosphatase is regulated, then a similar mechanism might be responsible for pyrophosphatase regulation. Otherwise, the Mg^{2+}–pH dependence might be an indication of other, more specific allosteric properties. In any event, any regulation of pyrophosphatase in chloroplasts would be effected by a different mechanism than that found in other systems, since the activity of the enzyme in chloroplasts is unaffected by ATP, ADP, or AMP.

PP_i added to a suspension of isolated spinach chloroplasts stimulates the photosynthetic reduction of CO_2 (refs. 24, 30). The inhibitory effects on such fixation of factors isolated from spinach leaves depends on the ratio of added pyrophosphate to added factors[31]. The level of ^{32}P-labeled pyrophosphate in *Chlorella pyrenoidosa* which have been photosynthesizing in the presence of $^{32}P_i$ changes suddenly during the transition from light to dark[32], and also in the light upon the addition of fatty acids which are known to cause changes in the activity of regulated enzymes of the carbon reduction cycle and inhibition of photophosphorylation[33]. Furthermore, the addition of octanoic acid causes a transient increase in pyrophosphate at the same time that the level of ATP declines and while the synthesis of carbohydrates and PP_i in the chloroplasts must be presumed to decrease.

The presence of PP_i in green tissue at levels comparable to those of other metabolites suggests that the activity of inorganic pyrophosphatase may be limited, and thus possibly subject to regulation. The rise in PP_i level, along with the inactivation of

two regulated enzymes, fructose diphosphatase and phosphoribulokinase, upon addition of octanoic acid also suggests pyrophosphatase regulation. Since PP_i itself appears to affect other biochemical activities, control of its level through the action of a regulated pyrophosphatase could be part of a feedback control, from carbohydrate synthesis to CO_2 fixation rate, or through the distribution of carbon fixed by the carbon reduction cycle to non-carbohydrate biosynthesis.

It seems likely that properties of pyrophosphatase enzyme in some tropical grasses (maize, sugar cane, *etc.*) which contain a special CO_2 fixing pathway[34] may be different from properties of the enzyme in spinach and the majority of green plants which do not contain this special pathway. Such a difference would be due to the fact that PP_i is formed in one step of that special pathway, during the pyruvate inorganic phosphate dikinase reaction[35]. Nevertheless, the properties of the purified enzyme isolated from spinach chloroplasts are similar in some respects to properties described for pyrophosphatase activity in a homogenate of maize leaves[20].

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