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THE DISTRIBUTION AND PROPERTIES OF ALKALINE INORGANIC PYROPHOSPHATASE FROM HIGHER PLANTS

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Abstract—An alkaline inorganic pyrophosphatase (E.C. 3.6.1.1) has been detected in a range of species and is associated at least partly with the chloroplast fraction. It is as active in plants showing only the carbon reduction cycle of carbon dioxide fixation as in plants exhibiting the 4-carbon acid pathway. The enzyme has been isolated and partially purified from sugar cane leaves. It has an absolute requirement for magnesium ions and shows a pH optimum of 8·3. It is inhibited by orthophosphate, arsenate and EDTA but is unaffected by —SH group inhibitors. Possible functions of the pyrophosphatase are discussed.

INTRODUCTION

A 4-CARBON acid pathway of carbon dioxide fixation in sugar cane and a number of other species has been demonstrated.[1-3] A key enzyme in this pathway, pyruvate phosphate dikinase (E.C.2.7.1),[4,5] can produce sufficient phosphopyruvate to maintain the observed rates of CO_2 fixation via phosphopyruvate carboxylase (4.1.1.31) only if the other products of the reaction, pyrophosphate and AMP, are removed rapidly. Recently Hatch, Slack and Bull[6] and Simmons and Butler[7] have suggested that an inorganic pyrophosphatase (3.6.1.1) (PPase) is present in high activities only in species exhibiting the 4-carbon acid pathway.

Present studies suggest that the PPase enzyme is not restricted in its occurrence only to these species. The enzyme has been partially purified from sugar cane leaves and its properties studied.

RESULTS AND DISCUSSION

Comparative Studies

Table 1 summarizes the occurrence and specific activity of the alkaline inorganic PPase in a range of species. Generally, the activity is highest in the tropical grasses (sugar cane, *Sorghum*, *Panicum* and maize), but spinach has a specific activity inferior only to sugar cane and maize. The specific activities are of the same order as those described by Hatch *et al.*[6] The range of activities suggests that there is no clear-cut difference in the PPase activity between species showing the 4-carbon acid cycle and other species. Comparison with the PEP carboxylase activity of the samples (Table 1) underlines this; of the species other than tropical

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TABLE 1. ASSAY OF PPASE AND PEP CARBOXYLASE ACTIVITIES OF A RANGE OF PLANTS

Tissue	Protein (μg/100 μl)	PPase activity (mμmoles Pi released/mg protein/min)	PEP carboxylase (counts/100 sec/mg protein/min)
Sugar cane	79	4936	1632
Urtica dioica L.	705	199	ND
Spinacia oleracea	38	3684	ND
Filipendula ulmaria	140	757	ND
Aloe spp.	230	639	10
Maize	290	4500	64
Panicum fasciculatum	600	1038	157
Sorghum	435	1353	738
Opuntia	82	488	164
Chenopodium album	150	4560	78

ND = None detected.

Protein was extracted from leaf samples by the method of Hatch *et al.*[6] Reaction mixtures for PPase assay contained (in μmoles) PPi 5; $MgCl_2$ 20; tris-HCl, pH 8·3,350; protein 100 μl, assay as described in Experimental. PEP carboxylase was assayed as described in that section.

grasses, only *Opuntia* has measurable PEP carboxylase activity. It has been suggested[8] that *Opuntia* species show the 4-carbon acid pathway.

Intracellular Distribution of PPase

This was studied in sugar cane, spinach and *Chenopodium album* using a simple medium which did not retain chloroplast integrity. Table 2 summarizes the results; in each species the chloroplasts show PPase with specific activity higher than in the 25,000*g* fraction, suggesting that at least a portion of the total PPase originated in the chloroplasts. The bulk of the enzyme is associated with the final supernatant but a high-speed fraction shows PPase activity. These results are compatible with the suggestions that PPase is concentrated in the chloroplasts[7] and that it is associated with photophosphorylase particles[9] which are readily

TABLE 2. DISTRIBUTION OF PPASE BETWEEN SUBCELLULAR FRACTIONS OF SPINACH, SUGAR CANE AND FAT HEN (*Chenopodium album* L.) LEAVES

	Chloroplasts		25,000 *g*		170,000 *g*		Supernatant	
Species	Protein (mg)	Activity mμmoles/mg protein/min	Protein	Activity	Protein	Activity	Protein	Activity
Spinach	37	1100	48	102	11	742	255	5300
Sugar cane	41	980	72	495	20	5804	193	6369
Fat Hen	43	689	84	609	45	816	173	4760

Chloroplasts were prepared as described in the text, the supernatant was centrifuged at 25,000 *g* for 20 min and the supernatant from this spin centrifuged at 170,000*g* for 2 hr. PPase activities were determined at pH 8·3 as described in the text.

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[9] A. E. KARU and E. N. MOUDRIANAKIS, *Arch. Biochem. Biophys.* **129**, 655 (1969).

released from the chloroplasts. Diluting the chloroplast preparations and sonicating briefly released all the PPase activity; this could be recovered from the supernatant after centrifugation of the sonicate at 50,000*g* for 15 min.

pH Optimum

The activity of PPase was measured over a pH range from 4·0 to 9·3 using reaction mixtures containing either no $MgCl_2$ or 20 μmoles of $MgCl_2$ and 250 μmoles of β-alanine–acetate, MOPS–NaOH or tris–HCl buffer. Table 3 summarizes the results; in the presence of magnesium the pH optimum was 8·3, without magnesium the pH optimum was 5·5 and the activity much lower. The pH optima are similar to those described[10] for PPase from potato tubers, but the distribution of activity between the two is very different.

TABLE 3. EFFECT OF pH ON THE ACTIVITY OF CANE LEAF PPASE IN THE PRESENCE AND ABSENCE OF Mg^{2+} IONS

pH	Buffer	Rate (mμmoles/mg protein/min)	
		+ Mg^{2+}	− Mg^{2+}
4·0	β-Alanine–acetate	46	40
4·3		29	24
4·6		27	72
4·9		132	121
5·2		164	40
5·5		168	201
5·8		240	193
6·0		247	152
7·0	MOPS–NaOH	606	88
7·1	Tris–HCl	884	64
7·7		1300	32
8·3		1383	24
8·7		1290	16
9·3		886	8

Reaction mixtures contained (in 0·7 ml) 5 μmoles PPi, 20 μmoles $MgCl_2$, 350 μmoles buffer and 100 μl (445 μg) cane leaf protein.

Effect of Mg^{2+}

No enzyme activity could be detected at pH 8·3 with less than 2·5 μmoles of $MgCl_2$ in the reaction mixture (i.e. $1Mg^{2+}$:2PPi) (Table 4). There was only a comparatively small increase in activity when the Mg^{2+} level was increased above 20 μmoles ($4Mg^{2+}$:1PPi). These results agree well with those of Bloch-Frankenthal[11] who showed an optimum Mg^{2+}:PPi ratio of 3·81 for a PPase isolated from red blood cells and concluded that the substrate of the enzyme was probably the complex ion $(MgP_2O_7)^{2-}$.

Metal Ion Specificity

The effects of various divalent metal ions as co-factors for PPase at pH 8·3 at the level of $2M^{2+}$:1PPi are summarized in Table 5. None is as effective as magnesium.

[10] B. NAGANNA, A. RAMAN, B. VENUGOPAL and C. E. SRIPATHI, *Biochem. J.* **60**, 215 (1955).
[11] L. BLOCH-FRANKENTHAL, *Biochem. J.* **57**, 87 (1954).

TABLE 4. EFFECT OF Mg^{2+} CONCENTRATION ON THE ACTIVITY OF CANE LEAF PPASE

μmoles $MgCl_2$/reaction mixture	Mg^{2+}:PPi (mole/mole)	Activity (% of maximum)
0	0	0
0.5	0.1	0
1.0	0.2	0
2.5	0.5	6.2
5.0	1.0	50·0
10.0	2.0	78.0
20.0	4.0	100·0

Reaction mixtures contained (in 0·7 ml) 5 μmoles PPi, 350 μmoles tris-HCl (pH 8·3), and 100 μl of cane leaf protein (445 μg protein). PPase activity was determined as described in Experimental.

TABLE 5. EFFECTS OF VARIOUS DIVALENT METAL IONS AS ACTIVATORS OF PPASE

Metal ion	Reaction rate (mμoles Pi released/mg protein/min)	Enzyme activity (% of maximum)
Mg^{2+}	2393	100
Ca^{2+}	0	0
Zn^{2+}	56	2·3
Mn^{2+}	112	4·7

The assay was as in Table 1, but using 20 μmoles of the metal chlorides.

Purification of PPase

A sample of the crude cane leaf protein was chromatographed on a column of DEAE cellulose. The PPase activity was associated with a major protein peak which showed various enzyme activities, including malate dehydrogenase, PEP carboxylase and malic enzyme. The specific activity was increased three-fold.

The most active fractions were applied to a column of Sephadex G-200 which was eluted with 0·1 M tris overnight. This treatment purified the enzyme a little further, increasing the specific activity by 50 per cent and removing a brown contaminant. The most active fractions were bulked and used in further experiments.

Michaelis Constant

The relationship between enzyme activity and substrate concentration was determined at pH 8·3 using a constant ratio of Mg^{2+}:PPi of 4; a Lineweaver–Burk plot indicated a K_m of 0·75 mM. This compares with a K_m of 0·54 mM for the enzyme from erythrocytes;[11] Naganna *et al.*[10] did not maintain a constant Mg^{2+}:PPi ratio and were unable to obtain a value for the K_m and Simmons and Butler[7], using an excess of $MgCl_2$ found a K_m of 0·0056 mM for maize PPase.

Action of Inhibitors

PPase was not inhibited by iodoacetamide or *p*-chloromercuribenzoate even at concentrations as high as 50 mM and 5 mM respectively. EDTA inhibited at concentrations sufficient to complex the magnesium in the reaction mixture (Table 6). The enzyme was not

inhibited by sulphate at 50 mM. Orthophosphate inhibited the PPase, the inhibition being complete when the ratio of orthophosphate to PPi was 1·0 and arsenate was also inhibitory (Table 6).

TABLE 6. EFFECT OF INHIBITORS ON SUGAR CANE PPASE

Inhibitor	Concentration (mM)	PPase activity (% control)
Arsenate	3·0	58
	10·0	35
	20·0	16·5
	50·0	4·7
Orthophosphate	0·7	96·2
	1·4	85·0
	2·9	58·2
	4·3	35·5
	5·7	16·3
	7·1	0·0
EDTA	1·4	65·0
	2·1	34·0
	2·9	6·6
	3·6	1·0
	4·3	0·0

Reaction mixtures contained (in addition to the inhibitor) 5 μmoles PPi, 20 μmoles $MgCl_2$, 350 μmoles tris-HCl, pH 8·3, and 0·1 ml enzyme preparation (13·7 μg protein) in a total volume of 0·7 ml.

DISCUSSION

The alkaline inorganic PPase isolated from sugar cane is similar in many respects to the PPase isolated from yeast,[12] erythrocytes,[11] potato tubers,[10] moulds,[13] rat liver,[14] a chrysophycean flagellate[15] and maize leaves.[7] Bailey and Webb,[12] using the yeast enzyme, reported a pH optimum of 6·9 and found inhibition by iodoacetate. The present enzyme has a considerably higher pH optimum and is insensitive to —SH group inhibitors. It is closely similar to the alkaline PPase described by Naganna *et al.*,[8] Ricketts,[15] and Simmons and Butler.[7]

Naganna, Venugopal and Sripathi[16] assayed PPase in a wide range of photosynthetic tissues but unfortunately did not include any species now known to show the 4-carbon acid pathway. Using the data supplied, their activities can be compared only approximately with those measured here and by Hatch *et al.*,[6] but fall within a similar range (40–500 mμmoles of Pi released/min/0·025 g leaf).

The presence of PPase may explain the increase in activity of spinach chloroplasts after isolation in a medium containing PPi.[17] These chloroplasts were stored in a medium containing $MgCl_2$, so that it is possible that PPi carried over from the grinding medium might be hydrolysed slowly to produce a small amount of orthophosphate, sufficient to stimulate

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[14] R. C. NORDLIE and M. A. LARDY, *Biochem. Biophys. Acta* **50**, 189 (1961).
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[16] B. NAGANNA, B. VENUGOPAL and C. E. SRIPATHI, *Biochem. J.* **60**, 224 (1955).
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CO_2-dependent oxygen evolution. The observations of Baltscheffsky[18,19] that PPi acts as an energy donor in chromatophores of *Rhodospirillum rubrum*, which show high PPase activities, suggest that PPi may function similarly in chloroplasts, and indeed Vose and Spencer[20] have demonstrated that PPi stimulates CO_2 fixation by isolated spinach chloroplasts. The Mg^{2+} concentration within the chloroplast may control the activity of PPase; the studies of Stocking and Ongun[21] suggest that a large proportion of the total leaf Mg^{2+}, certainly sufficient to support PPase activity, is located in the chloroplasts.

Since Mg^{2+} ions are apparently transferred from thylakoids to the stroma on illumination of chloroplasts,[22] the consequent changes in concentration may control the activity of PPase *in vivo* in a manner similar to those suggested for alkaline fructose diphosphatase[23] and ribulose diphosphate carboxylase.[24] Such light-induced changes in Mg^{2+} ion distribution might account for the absence of dark CO_2 fixation in sugar cane leaves.[1] A dark uptake of Mg^{2+} into the thylakoids[22] might withdraw sufficient Mg^{2+} to inactivate PPase in the cytoplasm or in the stroma. In turn this might inactivate the pyruvate; Pi dikinase and prevent the regeneration of PEP, preventing CO_2 assimilation via PEP carboxylase.

The activity of PPase in the species studied suggests that it is probably of importance to photosynthesis in all plants rather than merely in those showing the 4-carbon acid pathway of CO_2 assimilation.

EXPERIMENTAL

Plant Materials

All plant material was harvested immediately before use. Sugar cane (*Saccharum* hybrid) was grown in a heated greenhouse maintained above 10° without supplementary illumination. *Zea mays*, *Sorghum* hybrids and *Panicum fasciculatum* were grown at 25° in a regime of 13 hr light (11,000 lux) and 11 hr dark. Spinach (*Spinacia oleracea*) was grown in a cool greenhouse. *Opuntia microsperma albida* was a single potted plant purchased locally. Other plants were collected from the field.

Extraction of Leaf Protein

For the extraction of protein from small amounts of tissue the method of Hatch *et al.*[6] was used. Certain species, e.g. a *Kalanchoe* and *Saxifraga spathularia* × *umbrosa* (London Pride) could not be extracted successfully by this method owing to the presence of tannins which precipitated most of the protein.

For the larger-scale preparation of PPase, 50 g of cane leaves were washed with distilled water, sliced into 1 cm segments and comminuted in a partly frozen slurry containing 1% (w/v) NaCl, 0·05% (w/v) β-mercaptoethanol and sufficient tris buffer to give pH 7·0 after grinding. The brei was filtered through eight layers of muslin and the filtrate centrifuged at 40,000g for 20 min. The amber supernatant was saturated with $(NH_4)_2SO_4$, left to stand for at least 30 min, and the precipitated protein harvested by centrifugation. All procedures were carried out at 4°. Protein was determined by the method of Lowry *et al.*[25] using bovine serum albumin as a standard.

Chloroplast protein was isolated by precipitation with 80% acetone (v/v) and washed with further 80% acetone to remove lipids and also endogenous phenols which may interfere with the assay.

Enzyme Assay

PPase was assayed using the method of Hill and Walker[26] to determine the orthophosphate formed from pyrophosphate during incubation at 30°. Reaction mixtures contained 5 μmoles of PPi in a total final volume of 0·7 ml; other components were varied as described in the text. Reactions were started by adding the extract

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to be assayed and stopped by the addition of 9·5 ml of perchloric acid-metabisulphite-amidol reagent.[26] At the concentration used, PPi did not interfere with the estimation of orthophosphate.

PEP carboxylase was assayed by measuring the radioactivity incorporated in to acid-stable compounds in a reaction mixture containing (in μmoles) PEP 1; glutamate 10; $MgCl_2$ 1; $NaHCO_3$ (containing 2 μ ^{14}C) 1; tris-HCl, pH 8·3, 110, and 0·1 ml protein preparations; total volume 0·4 ml. Reactions were run at 30° and stopped by adding 0·1 ml of 24% (w/v) trichloroacetic acid.

Chloroplast Preparation

Chloroplasts were isolated by a modification of the method of Walker[27] in 0·33 M sucrose containing 0·01 M MOPS–NaOH, pH 7·4. This was chosen so that no Mg^{2+} or other metal ions were added until PPase was assayed: sucrose was preferred to sorbitol because the latter interferes with the determination of orthophosphate.[28] Chlorophyll was determined by the method of Bruinsma.[29]

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