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ALKALINE INORGANIC PYROPHOSPHATASE FROM ORCHID LEAVES

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Key Word Index—*Aranda* Christine 130; Orchidaceae; orchid; alkaline inorganic pyrophosphatase; purification; properties.

Abstract—An alkaline inorganic pyrophosphatase (IP) from leaves of an orchid, *Aranda* Christine 130 (*Arachnis hookerana* var. *luteola* × *Vanda* Hilo Blue) was purified by acetone precipitation and chromatography on Sephadex G-75 and DEAE-cellulose. The IP gave a single band on non-denaturing gel electrophoresis at pH 8.3 and its M_r determined by gel filtration, was 28 000. The pH optimum was 9 and the IP required Mg^{2+} for its activity and stability. The IP exhibited high specificity for PPi and attained a maximum activity at a Mg^{2+}:PPi ratio of 10:1. Other cations tested could not replace Mg^{2+} and they were also found to be inhibitory. The IP was also inhibited by EDTA and F^- but not by iodoacetamide.

INTRODUCTION

Plants with Crassulacean acid metabolism (CAM) fix CO_2 initially via phosphoenolpyruvate but do so primarily at night [1]. This reaction leads to the accumulation of malate in CAM tissues. Present evidence indicates that storage carbohydrates such as starch and polyglucose may be important sources of carbon for the synthesis of phosphoenolpyruvate needed for CO_2 fixation at night [2]. During the day, phosphoenolpyruvate is regenerated as a result of malate decarboxylation and is most probably metabolised to starch or other storage carbohydrates via gluconeogenesis [3]. Inorganic pyrophosphate is a substrate or product of a number of key reactions involved in the biosynthesis of starch and other carbohydrates [1, 3, 4]. Thus, enzymes which hydrolyse pyrophosphate may play an important role in regulating the metabolism of malate in CAM tissues. The presence of an alkaline inorganic pyrophosphatase (IP) (EC 3.6.1.1) which hydrolyses pyrophosphate specifically has been well documented in various plants [5–7]. The enzyme has also been partially purified from a number of C_3 and C_4 plants and studied in relation to photosynthesis [8–16]. However, relatively little is known about the enzyme in CAM tissues. The occurrence of the enzyme in *Aranda* Christine 130, a tropical orchid with a CAM mode of CO_2 fixation, has been reported [17]. Here, we report the isolation and purification of the enzyme from the leaves of this plant and compare its properties with IPs obtained from various other C_3 and C_4 plants.

RESULTS AND DISCUSSION

Orchid leaves usually possess large amounts of organic acids, alkaloids, anthocyanins and diphenol oxidases. These interfering materials present great problems in the extraction and purification of enzymes and there have been few enzymatic studies on tropical orchids, particularly those with very thick leaves [17]. *Aranda* Christine 130 was chosen in the present study because of its abundance in Singapore. This makes it a good candidate for the purification of proteins. Furthermore it has already been used in studies related to photosynthesis [17].

The procedure described here and summarized in Table 1 represents optimal conditions for the extraction and purification of the alkaline IP from thick leaves of *Aranda* Christine 130. Grinding with mortar and pestle proved to be the best way to extract most of the enzyme

Table 1. Purification of an alkaline inorganic pyrophosphatase from orchid leaves

Fraction	Volume (ml)	Total protein (mg)	Total activity (nkats)	Specific activity (nkats/mg protein)	Recovery (%)
Crude extract	2500	—	717.5	—	100
Acetone precipitation	82.5	—	259	—	36
Sephadex G-75	25	6.2	95.5	15.5	13.3
First DEAE cellulose	20	3	90	30	12.5
Second DEAE cellulose	5	1	45	45	6.3

from the leaves. However, the enzyme was unstable during the purification procedure, particularly at the Sephadex G-75 chromatography step and also during its storage in frozen state. Addition of 10 mM magnesium chloride to the enzyme solution produced the most effective stabilizing effect. This is in common with the observations of the enzyme from corn leaves [9]. Treatment of the crude extract with acetone as 50% saturation was the most reliable method for removing many of the interfering compounds like phenols and alkaloids from the precipitated enzyme. Ammonium sulphate precipitation was not recommended because it induced the enzyme to aggregate into a sticky yellow mass which also failed to precipitate under high speed centrifugation. Fractionation of the acetone pellet with Sephadex G-75 resulted in the separation of the enzyme from most of the diphenol oxidases. Further purification on DEAE columns yielded a 6.3% recovery of the total activity and a sp. act. of 45 nkat/mg protein. The purity of the enzyme preparation was checked by non-denaturing gel electrophoresis at pH 8.3. The electrophoretic pattern revealed one main protein band after staining with Coomassie Blue. A separate gel, which was stained for enzyme activity, showed one intense yellow band with an R_f value similar to that of the protein band. The M_r of the purified enzyme determined by gel filtration was 28 000. The present enzyme is somewhat smaller than those of C_4 plants. Values of 38 000 and 32 860 have been reported for alkaline IPs from maize [14] and *Amaranthus* [16] leaves, respectively.

The enzyme was highly specific for PP_i. Other substrates when tested under similar conditions failed to react with the enzyme. These included β-glycerophosphate, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1, 6-diphosphate, *p*-nitrophenol phosphate, ATP, ADP and AMP. This indicates that the enzyme is free from other phosphomonoesterases or phosphodiesterases.

Determination of the pH activity profile of the enzyme was conducted in reaction mixtures containing either no magnesium chloride or 10 mM magnesium chloride. Without magnesium chloride, no enzyme activity was obtained over a pH range from 4 to 10. In the presence of magnesium chloride, the enzyme showed a pH optimum at 9.0 and its activity became negligible in the acidic range. These findings indicate that the enzyme was free of any acid IP activity. In contrast, alkaline IPs from guar cotyledons [15], potato [18], corn endosperm [19], sugarcane leaves [5] and corn leaves [14] were reported to have acid IP activities. The pH optimum of the present enzyme is comparable with those reported for alkaline IPs from different plant tissues in a variety of species [8–16]. It has also been reported that the pH optimum of the enzyme from spinach chloroplast [12], maize leaves [14] and Dubh grass [20] varies with magnesium chloride concentrations. Thus, the effect of magnesium chloride on pH optimum of the present enzyme was investigated. This was conducted by measuring the enzyme activity in 1 mM PP_i at different pH using Mg^{2+} : PP_i ratios of 1:1, 3:1, 5:1, 10:1 and 20:1 (Fig. 1). The pH optimum for the enzyme activity was 9 at different Mg^{2+} : PP_i ratios. The maximum activity was achieved at a 10:1 ratio. This ratio is identical to those reported for the alkaline IPs from sugarcane cell walls [21] and *Amaranthus* leaves [16] but somewhat greater than those obtained for the alkaline IPs from corn [9] and maize [22] leaves. In addition, the present enzyme was not inhibited even at a ratio of 20:1. This is not unusual since the enzyme becomes more stable in the presence of Mg^{2+} ions, as described earlier. Mg^{2+} ions may also combine with PP_i ions to form the actual substrate; this is supported by the finding that an excess of PP_i over Mg^{2+} inhibited the enzyme activity markedly.

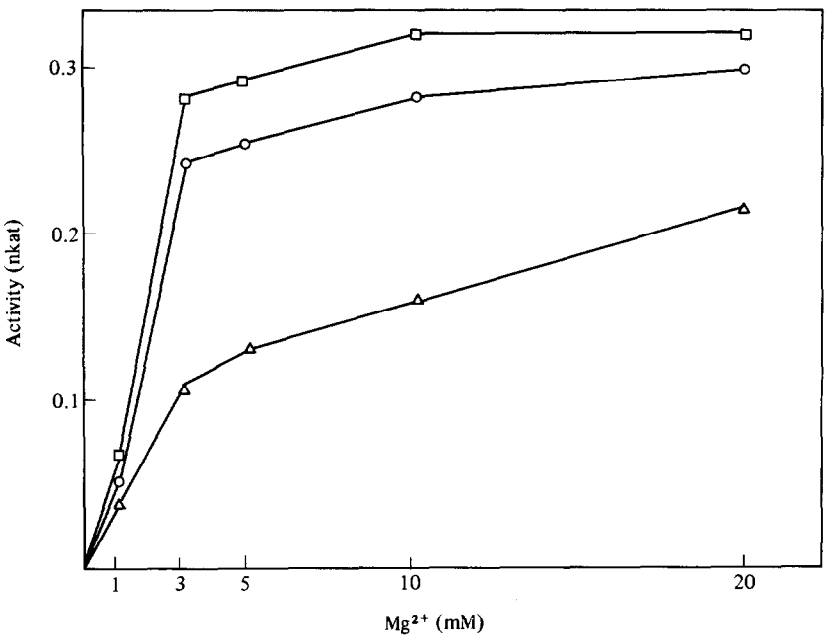

Fig. 1. Effect of $MgCl_2$ concentration on PPi hydrolysis at different pH values: pH 9 (□); pH 8.5 (○); and pH 7.3 (△).

The Mg^{2+} and PP_i ions are likely to combine as $MgP_2O_7^{2-}$ and $Mg_2P_2O_7^0$ at pH 9.0 [23]. Since the enzyme works best at high Mg^{2+} concentrations, the most likely substrate is $Mg_2P_2O_7^0$.

The enzyme has an absolute requirement for Mg^{2+}. None of the other cations tested, K^+, Na^+, Ca^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Fe^{3+} could activate the enzyme. The effect of several mono-, di- and trivalent cations on the enzyme activity in the presence of 10 mM Mg^{2+} was also investigated (Table 2). All cations except Na^+ and K^+ inhibited the enzyme activity significantly at 5 mM concentrations. Na^+ and K^+ ions were inhibitory only at 10 mM concentration. Among the cations, Ca^{2+} was the most effective inhibitor. It reduced the enzyme activity by half at 1 mM concentration. Chemicals reported to have an inhibitory effect on the alkaline IPs from other plants [5, 10, 15] were also tested with the present enzyme. These included F^-, EDTA, sodium citrate, sodium cyanide, sodium arsenate and thiourea. Complete inhibition occurred with F^- and EDTA at 1 and 10 mM concentrations, respectively. All the other compounds failed to inhibit the enzyme even at 10 mM concentration. The enzyme was not inhibited by the thiol reagent, iodoacetamide, at 10 mM concentration.

The results described show clearly that the properties of the purified enzyme isolated from a CAM plant are similar to properties described for alkaline IPs from various C_3 and C_4 plants.

EXPERIMENTAL

Aranda Christine 130 plants were grown naturally in the garden of the Botany Department, National University of Singapore. For preparation of the alkaline IP, healthy leaves (500 g) were excised from the centre of shoots in the morning. All preparations were carried out at 4°. Leaves in batches of 50 g were hand-ground into small pieces with mortar and pestle and then thoroughly ground in 250 ml of a medium containing 50 mM Tris base, 2 mM EDTA, 6 mM Na diethyldithiocarbamate, 25 mM Na ascorbate and 5 g PVP. The crude extract was filtered through several layers of cheesecloth to remove the coarse debris. It was then centrifuged for 10 min at 30000 *g* to give a yellow supernatant. To this supernatant Me_2CO, previously chilled to −20°, was added to 50% and the pellet was collected by centrifugation. This pellet was resuspended in a small volume of a Tris buffer containing 50 mM Tris–HCl (pH 7.5) and 10 mM $MgCl_2$, and then dialysed overnight with two changes of the Tris buffer. After dialysis, the resuspension was centrifuged to remove insoluble materials. The supernatant was concd by ultrafiltration before passing it through a Sephadex G-75 column (96 × 3 cm) washed with the Tris buffer. The active fractions were loaded on to a DEAE-cellulose column (9 × 2 cm) previously equilibrated with the Tris buffer. The column retained a lot of yellow materials but not the enzyme. The active eluate was dialysed overnight against two changes of a second Tris buffer containing 5 mM Tris–HCl (pH 7.5) and 1 mM $MgCl_2$. The dialysed material was applied on to a DEAE-cellulose column (18 × 2 cm) previously equilibrated with the second Tris buffer. A linear gradient was established with increasing concn of NaCl (0–0.2 M) prepared in 50 mM Tris–HCl (pH 7.5) and 10 mM $MgCl_2$. Fractions containing the enzyme were pooled for subsequent studies. Unless otherwise stated, the enzyme was assayed at 25° for 15 min in 1 ml of the reaction mixture containing 1 mM PP_i, 100 mM Tris–HCl (pH 9.0) and 10 mM $MgCl_2$. The P_i released was measured as described in ref. [24].

Non-denaturing gel electrophoresis were performed according to the method of ref. [25]. Gels were stained either for protein by Coomassie Brilliant Blue G250 or enzyme activity according to ref. [26].

M_r determination of the enzyme was carried out on a Sephadex G-75 column (48 × 1.5 cm) in 100 mM Tris–HCl (pH 7.5) and 10 mM $MgCl_2$. The M_r standards were cytochrome *c*, α-chymotrypsinogen, ovalbumin and albumin.

Table 2. Effect of cations on alkaline inorganic pyrophosphatase

	Concentration (mM)		
	1	5	10
Cations	Activity (% of control)		
Na^+	100	98	85.7
K^+	100	100	82.1
Mn^{2+}	82	22	8.9
Cu^{2+}	71	50	8.9
Zn^{2+}	82	50	8
Ca^{2+}	42	4.2	4
Co^{2+}	83	53	53
Fe^{3+}	98	7	7

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