PARTIAL PURIFICATION AND CHARACTERISATION OF ACID PHOSPHATASES FROM DEVELOPING CHICKPEA SEEDS

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Received on 01st Jan., 2011, Revised and Accepted on 06th May, 2011

SUMMARY

Activity of acid phosphatases in chickpea seeds was found to be highest at the early stage of pod development (14 days after flowering). Acid phosphatases were therefore purified from seeds at early stage by DEAE-cellulose column chromatography followed by gel permeation chromatography, as three partially purified forms which were designated as acid phosphatase-1, acid phosphatase-2 and acid phosphatase-3. These forms were separately studied for their kinetic properties. All three forms had optimum pH of 5.5, optimum temperature of 55-60°C but differed in substrate specificity. Pyrophosphate and p-nitrophenylphosphate were good substrates, phytic acid poor substrate and 3-phosphoglycerate and adenosine-tri-phosphate differed for their preference for all three forms of enzyme. Activities of these forms respond differently to various metal ions and chelating agents.

Key words: **Chickpea, legume, phosphatases, purification**

INTRODUCTION

Phosphatases catalyse the hydrolysis of phosphate monoesters and are classified as acid and alkaline based solely upon their optimal activity at pH below or above 7 (Vincent *et al.* 1992). Plant alkaline phosphatases are characterised to be more specific with their specific metabolic routes while acid phosphatases have been shown as nonspecific in nature and more studied in plants (Duff *et al.* 1994). Acid phosphatases are ubiquitous in plants, animals, fungi, and bacteria. They function in the production, transport and recycling of Pi which is crucial for cellular metabolism and energy transduction processes. They exist as tissue and/or cellular compartment specific-isozymes that display variations in subunit molecular weights, substrate specificity and sensitivity to inhibition by various ions and metabolites. Acid phosphatases have been purified from leaves (Garcia *et al.* 2004), seeds (Olczak *et al.* 1997, Granjeiro *et al.* 1999), tubers (Kusudo *et al.* 2003), root nodules (Garcia *et al.* 2004), seedlings (Yenigun and Guvenilir 2003), coleoptiles (Pasqualini *et al.* 1997), cotyledons (Shekar *et al.* 2002, Gonnety *et al.* 2006, 2007), embryonic axis (Yoneyama *et al.* 2004). In roots, they might be involved in the solubilisation of macromolecular organophosphates in the soil so that Pi is made available to plants (Duff *et al.* 1994). In germinating seeds or seedlings, they might function to provide Pi to growing plant by hydrolysing organic phosphate reserves of the seeds (Duff *et al.* 1994). Otherwise, *in-vivo* function of plant acid phosphatases remains largely unknown though they are also found to be induced under various abiotic and biotic stresses (Plaxton and Carswell 1999) and under phosphate starvation (Bozzo *et al.* 2002). Alkaline phosphatases though less studied (Duff *et al.* 1994) are reported in root nodules of mungbean and chickpea (Gupta *et al.* 1998, Kaur *et al.* 1999). Studies on phosphatases in developing plant seeds are mostly lacking though they are studied in germinating seeds and seedlings. In this study, we analysed the activity pattern

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of acid phosphatases in chickpea seeds during pod development and partially purified them as three enzymatic forms by DEAE-cellulose column chromatography and gel permeation (Sephadex G-150) chromatography. These partially purified forms were studied for their kinetic properties.

MATERIALS AND METHODS

Materials: Chickpea (*Cicer arietinum* L. cv. GL-769) was used in the present investigation. The crop was sown in the fourth week of October. Developing flowers were tagged for 6-7 days and pods were collected at 14, 21, 28, 35 and 45 days after flowering (DAF) stages. Pods and seeds were separated on ice and brought under ice cold conditions to the laboratory.

Extraction and Assay of Enzyme Activities: Chickpea seeds (0.5 to 1g) were crushed in chilled pestle and mortar and extracted twice with 3 ml of 0.05 M sodium acetate buffer (pH 5.0). The pooled extract was passed through the double layered cheese cloth and centrifuged at 10,000 g for 15 minutes at 4^0C. The supernatant was passed through Sephadex G-25 column using the same buffer to remove inorganic phosphate and other low molecular weight components from crude extract (Kaur *et al.* 1999).

Enzyme activity was determined by using p-nitrophenyl phosphate as substrate where p-nitrophenol released after the action of phosphatase was directly read at 420 nm. Assay system consisted of 2.2 mM p-nitrophenyl phosphate, 50 mM sodium acetate buffer (pH 5.0) and 0.1 ml of enzyme in total volume of 3.1 ml at 37^oC. Reaction was stopped by adding 2 ml of 2N NaOH. Mixture was read at 420 nm against blank having 0.1 ml of double glass distilled water in place of enzyme preparation. Standard curve of different concentrations of p-nitrophenol was used. Protein content was measured by using Folin-phenol reagent (Kaur *et al.* 1999). Enzymes were extracted from three different samples and assayed in duplicate. Data are the mean ± S.D. of these values. Conditions of the assay system for enzyme were standardised to give linear rates with time with respect to substrate concentrations.

Purification of Acid Phosphatases: For purification of enzyme, the crude enzyme extract (at 4^0C) was precipitated with 2.5 volumes of chilled ethanol (about -15^0C) for overnight at -10^0C. Precipitates were centrifuged at 10, 000 g for 15 min and pellet was dissolved in a minimum volume of 0.05M sodium acetate buffer (pH 5.0). The enzyme was further purified by DEAE-cellulose column chromatography (Pharmacia low pressure column chromatography system) using linear gradient of 0-1 M where flow rate of 25 ml hr^{-1} was maintained with the help of peristaltic pump. Fractions of 5 ml each were collected and salt gradient was applied after Fraction 13. Enzyme was eluted as two peaks (Fig. 1A), peak which was obtained before applying gradient was called Peak-I and the other peak obtained after the gradient was designated Peak II. Fractions corresponding to both peaks were pooled separately and further purified by Gel permeation on Sephadex G-150 column. Enzyme preparation obtained from Peak I gave two peaks on gel permeation column called AP-1 and AP-2 (Fig. 1B) and enzyme obtained from Peak II gave only one peak, AP-3 (Fig. 1C). Ve/Vo was calculated by measuring the void volume of the column with dextran blue. All three enzyme fractions (AP-1, AP-2 and AP-3) were taken as partially purified acid phosphatases fractions and studied for their properties separately.

Characterisation of Acid Phosphatase Activity: The effect of pH on activity was assessed using 0.2M buffers of sodium citrate for pH from 3.0 to 6.0 and tris-maleate for pH from 6.0 to 8.0. Acid phosphatase activity was determined using PNPP as substrate (Fig. 2A).

The effect of temperature was studied by incubating reaction mixture at 30, 35, 40, 45, 50, 55, 60, 65 and 70^oC. Thermal stability of enzyme was done by preincubating enzyme at 50^oC and 60^oC for different times (0, 30, 60, 120 min). Enzyme activity without any treatment was considered as 100. For both experiments, enzyme activity was determined using PNPP as substrate (Fig. 2B and 3).

Substrate specificity of the enzyme was measured by taking different substrates (ATP, a-naphthyl acid

phosphate, 3-PGA, PPi, F1, 6-BP, Phytic acid, G6P, F6P and G1P) in 2.2 mM concentrations separately. Pi released was assessed at 820 nm by Ames method (Kaur *et al.,* 1999) (Fig. 4A). Standard curve of KH_2PO_4 was used for calculation.

Kinetic parameters (Km and Vmax) were determined from Lineweaver-Burk plots using different concentrations of substrate (Table 3). The inhibition or activation of acid phosphatase by various metal ions and chelating agent was assayed by adding 2 mM of each substance to enzymatic reaction mixture. The following ions were tested: Ni^{+2}, molybdate, Ca^{+2}, PO_4^{-3}, Zn^{+2}, F^-, Sn^{+2}, Mg^{+2}, Hg^{+2}, Na^+, K^+, Co^{+2} and EDTA. Enzyme activity was determined using PNPP as substrate. The results were expressed as relative percentage of activity with respect to control reaction without any additional salt (Fig. 4B). Ki values and kind of inhibition were determined from Lineweaver-Burk plots after measuring enzyme activity at two different concentrations of inhibitor (2 and 3 mM KH_2PO_4, 0.5 and 1.0 mM of $ZnCl_2$ and NaF, 1 and 2 mM of $CoCl_2$ and $SnCl_2$) with increasing concentrations (0.1 to 2 mM) of PNPP (Table 4).

All above assays are done in duplicates and values are calculated as mean ± S. E.

RESULTS

Activity pattern of acid phosphatases in seeds during pod development (Table 1) showed highest activity at the earliest stage of seed development (14 DAF), thereafter activity showed declining trend with development. Therefore, acid phosphatase from crude enzyme extract of seeds at 14 DAF was purified by ethanol precipitation followed by DEAE-cellulose column chromatography where linear gradient of 0-1 M NaCl was applied after fraction 13. Major activities were detected in fractions 3-8 obtained before application of gradient however, second peak was also obtained between fractions 18-21 after the application of gradient (Fig. 1A).

The acid phosphatases of both peaks were further purified separately on Sephadex G-150 column

Table 1. Specific activity of acid phosphatases in chickpea seeds during pod development

DAF	nkat mg^{-1} protein
14	12.5±2.1
21	3.74±0.41
28	2.91±0.11
35	0.23±0.03
42	0.25±0.03

Values are mean of triplicate extractions ± S. D. Activity is determined with p-nitrophenyl phosphate as a substrate

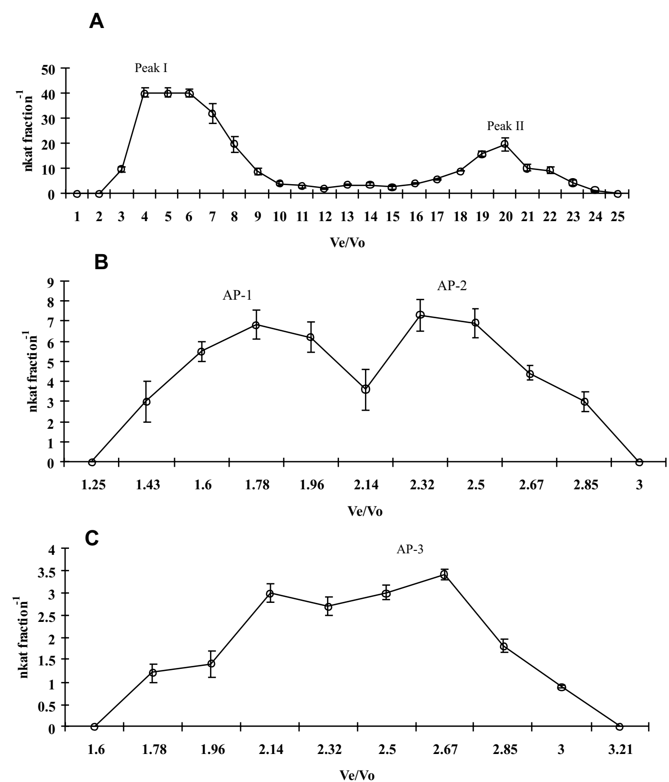

Fig. 1 (A). Elution profile of acid phosphatase obtained after ethanol precipitation on DEAE-cellulose column. A linear gradient of NaCl (0-1M) was applied from fraction 13. Fractions of 5 ml each were collected; (B). Elution profile of Peak-I obtained from DEAE-cellulose column chromatography on Sephadex G-150 column; (C). Elution profile of Peak-II obtained from DEAE-cellulose column chromatography on Sephadex G-150 column. Activity is measured using p-nitrophenyl phosphate as a substrate. Values are mean ± S.E. Specific activities were also measured in all fractions and similar profiles were obtained

chromatography. The pooled fraction obtained from fractions 4-7 gave further 2 peaks by Sephadex G-150 column chromatography (Fig. 1B). First peak was obtained around Ve/Vo 1.4 to 1.8 of maximum specific activity of 226.7 nkat mg^{-1} protein (Table 2) thus showing 30-fold rise in its specific activity over the crude extract. This enzyme was named as AP-1. Second peak was obtained between Ve/Vo 2.3 to 2.7 (Fig. 1B) with maximum specific activity of 60.8 nkat mg^{-1} protein hence 8-fold purified (Table 2) and was named as AP-2. The pooled fraction obtained from fractions 19 and 20 of DEAE-cellulose column chromatography when loaded on Sephadex G-150 column, it resolved into a single peak of maximum specific activity of 113.3 nkat mg^{-1} protein thus showing 15-fold (Table 2) rise in its specific activity over the crude extract and it was named as AP-3 (Fig. 1C). AP1, AP2 and AP3 represent mixtures of more than one enzyme, hence are more appropriately said to be partially purified acid phosphatases fractions.

Partially purified acid phosphatases (AP1, AP2, AP3) were studied for the effect of different ranges of pH (Fig. 2A) where all three acid phosphatase fractions (AP-1, AP-2, AP-3) exhibited maximum activity at pH

Table 2. Purification of acid phosphatase of seeds at 14 DAF

Steps of purification	Volume (ml)	nkat	Proteins (mg)	nkat mg^{-1}protein	Fold purification
Crude	90	2088	276	7.57	1.0
Ethanol precipitation	25	1267	68	18.6	2.5
DEAE-Cellulose (peak I)Fraction No.					
3.	5	9.6	0.5	19.2	2.5
4.	5	40.0	1.05	38.1	5.0
5.	5	40.0	1.2	33.3	4.4
6.	5	40.0	1.2	33.3	4.4
7.	5	32.0	1.05	30.5	4.0
8.	5	20.0	0.75	26.7	3.5
DEAE-Cellulose (peak II)Fraction No.					
18.	5	8.9	0.5	17.8	2.3
19.	5	16.0	0.75	21.3	2.8
20.	5	19.6	0.75	26.1	3.4
21.	5	10.3	0.5	20.6	2.7
Sephadex G-150 column (Acid phosphatase -1, AP-1)Ve/Vo					
1.6	5	5.5	0.03	183.3	24.2
1.78	5	6.8	0.03	226.7	29.9
1.96	5	6.2	0.06	103.3	13.6
Sephadex G-150 column (Acid phosphatase -2, AP-2)Ve/Vo					
2.32	5	7.3	0.12	60.8	8.0
2.50	5	6.9	0.18	38.3	5.1
2.67	5	4.4	0.12	36.7	4.8
Sephadex G-150 column (Acid phosphatase -3, AP-3)Ve/Vo					
2.32	5	2.7	0.03	90	11.9
2.50	5	3.0	0.03	100	13.2
2.67	5	3.4	0.03	113.3	15.0
2.85	5	1.8	0.03	60	7.9

5.5. Effect of temperature was studied by measuring the acid phosphatase activities over a temperature range of 30-70°C (Fig. 2B). Optimum temperature of AP-1 was found to be 55°C while optimum temperature of AP-2 and AP-3 was 60°C. Activities decreased rapidly at 70°C. Thermal stability of enzymes was studied by heating the enzyme preparations before measuring their activities for the period of 30 min, 60 min and 120 min at 50°C and 60°C (Fig. 3) where loss of 56-62%, 70-87%, 90% in the activities were observed for all three enzyme preparations (AP-1, AP-2 and AP-3A) respectively at 50°C when these enzyme preparations were heated for 30 min, 60 min and 120 min respectively (Fig. 3A). Pre-incubation at 60°C for 30 min resulted in 90% loss in the activities of all three enzyme preparations (Fig. 3B).

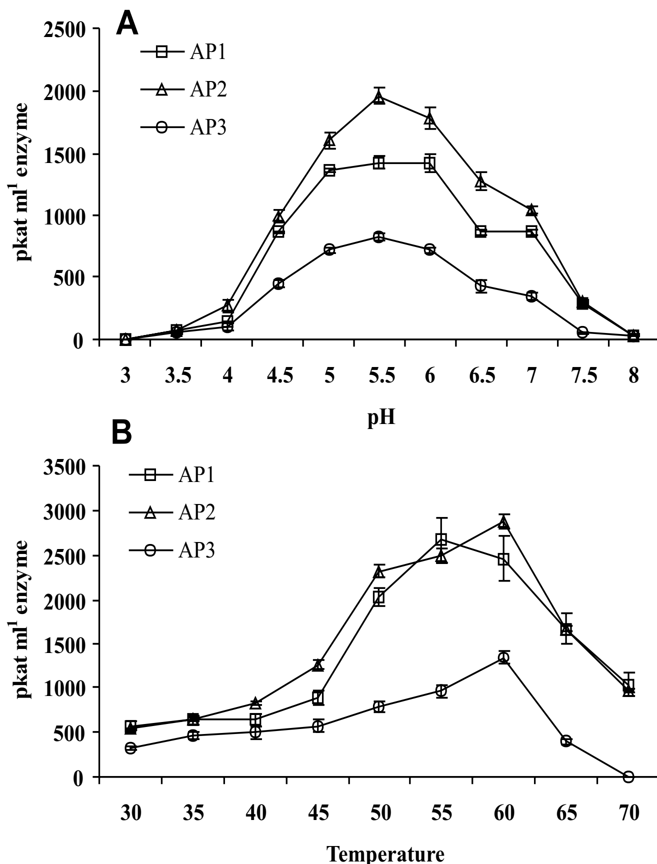

Fig. 2. (A). Effect of pH on the activity of AP-1, AP-2 and AP-3 fractions of acid phosphatase; (B). Effect of assay temperature on the activity of AP-1, AP-2 and AP-3 fractions of acid phosphatase. Activity is determined using p-nitrophenyl phosphate as a substrate. Values are mean ±S.E.

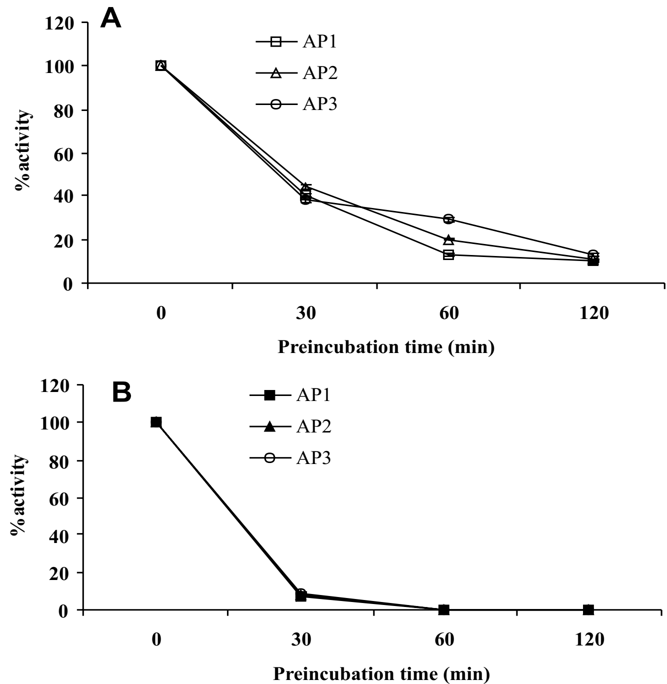

Fig. 3. Thermal stability of acid phosphatase fractions (AP-1, AP-2, AP-3) when preincubated at 50°C (in A) and 60°C (in B) before measuring the activity. Activity is measured with p-nitrophenyl phosphate as a substrate. The activity without heat treatment is 100. Values are mean ±S.E.

Substrate specificities were determined with different phosphorylated substrates (Fig. 4A) where PNPP was the synthetic substrate and others i.e. pyrophosphate (PPi), 3-phosphoglycerate (3-PGA), fructose 1,6-bisphosphate (FBP), adenosine-tri-phosphate (ATP), alpha-naphthyl acid phosphate (NAP), glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), phytic acid (PA) were natural substrates. All acid phosphatases fractions (AP-1, AP-2 and AP-3) showed broad acceptance of physiological substrates (Fig. 4A) with the highest rate against pyrophosphate (PPi). With PNPP, AP-1, AP-2 and AP-3 showed 42%, 51% and 46% of the activity in comparison to that observed with PPi respectively. For natural substrates, activity of AP-1 was in the order of PPi> 3-PGA> F-1,6BP> ATP > NAP> G6P > F6P > PA, order of AP-2 was PPi > ATP > F1, 6-BP > NAP > 3-PGA > F6P > G6P > PA and AP-3 was in the order of PPi > 3-PGA > ATP > NAP > F1, 6-BP > G6P > F6P > PA. Substrate specificity of AP-1 and AP-3 was almost similar. For AP-1 and AP-3, 3-PGA was a good substrate whereas 3-PGA, ATP, F-1,

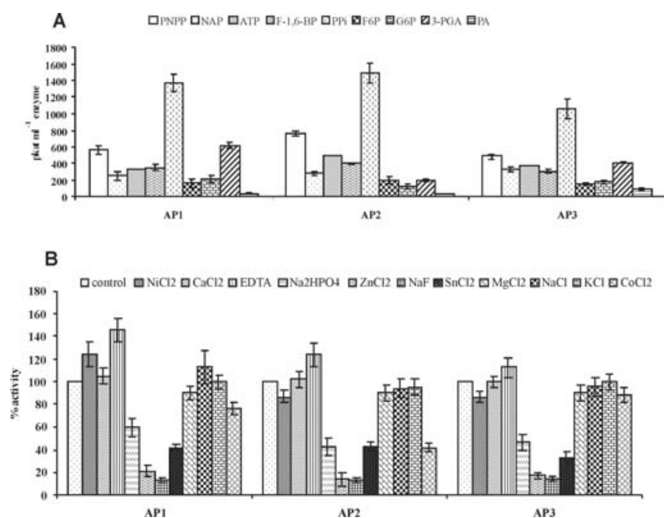

Fig. 4. (A). Substrate specificity of acid phosphatase fractions AP-1, AP-2 and AP-3 where different substrates used are PNPP, p-nitrophenyl phosphate; NAP, á-naphthyl acid phosphate; ATP, adenosine-tri-phosphate; F16BP, fructose 1,6-bisphosphate; PPi, pyrophosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; 3PGA, 3-phosphoglycerate; PA, phytic acid. Values are mean ±S.E. (B). Effect of various compounds (ions and chelating agent) on the activity of acid phosphatase (AP-1, AP-2, AP-3) where activities are given as relative % age of activity with respect to control reaction without any substance. Values are mean ±S.E.

6-BP were good in comparison to G6P, F6P and PA for AP-1 and AP-3. Phytic acid was the poorest substrate for all forms.

Four substrates 3-PGA, PPi, F1, 6-BP and PNPP were selected for studying the effect of their increasing concentration on the activity of acid phosphatases (Table 3). Km values of AP-1, AP-2 and AP-3 for PNPP were found to be about 0.33, 0.67 and 0.42 mM respectively. Km values for all three enzyme fractions with 3-PGA were found between 0.53 to 0.59 mM. With F-1, 6 BP, Km values were found to be in the range of 0.63 to 0.71 mM. The Km values of AP-1, AP-2 and AP-3 for PPi were 0.63, 0.67 mM, respectively. Highest Vmax was observed with PPi followed by PNPP and 3-PGA (Table 3).

Table 3. Effect of different substrates on Km and Vmax of AP1, AP2 and AP3 of acid phosphatases

Substrate	Km (mM)			Vmax (nkat ml^{-1} enzyme)		
	AP1	AP2	AP3	AP1	AP2	AP3
PNPP	0.33	0.67	0.42	0.33	0.33	0.24
3-PGA	0.59	0.59	0.53	0.28	0.42	0.21
F1,6BP	0.71	0.63	0.63	0.21	0.19	0.21
PPi	0.63	0.67	0.67	0.74	1.11	1.11

Various metal ions and chelating agent were studied for their effects on activities of AP1, AP2, AP3 fractions (Fig. 4B). These include Ni^{+2}, Ca^{+2}, Mg^{+2}, Na^{+}, K^{+}, Co^{+2}, Sn^{+2}, Zn^{+2}, Hg^{+2}as metal cations, F^{-}, PO^{-} ^{4}, molybdate ions as metal anions and EDTA as chelating agent. These were added in enzymatic assays at 2 mM concentration of their salt either in chloride forms (for metal cations) or sodium salt (for metal anions). The activities of these forms of enzyme responded differently to various metal ions. Hg^{+2} and molybdate ions were found to be strong inhibitors for all enzyme preparations as they completely inhibited the activity of all three enzymatic fractions (not shown in Fig. 4B). Ca^{+2}, Mg^{+2}, Ni^{+2}, Na^{+}, K^{+} had no effect on the activities of these acid phosphatases. EDTA was promotory for all three forms of acid phosphatases (Fig. 4B). Activation by EDTA was maximum for AP-1 (45%) followed by AP-2 (24%) and AP-3 (12%). Inhibition by Zn^{+2} and F^{-} ions was more than by PO_4^{-3} and Sn^{+2} ions. Zn^{+2} inhibited all forms almost equally. Inhibition by phosphate ions was greater for AP-2 and AP-3 than for AP-1. Co^{+2} did not affect AP-3. In contrast, Co^{+2} inhibited almost 60% of AP-2 activity and 24% of AP-1 activity.

For further studying the kinetics of inhibition, Zn^{+2}, Sn^{+2}, Co^{+2}, F^{-} and PO^{-4} ions were selected as these were observed to be significant inhibitors from the last experiment (Fig. 4B). $ZnCl_2$, NaF, $SnCl_2$ were found to be non-competitive inhibitors for AP-1 with Ki for $ZnCl_2$ being 0.16 mM, for NaF 0.28 mM and for $SnCl_2$ 1.47 mM (Table 4). NaF, $SnCl_2$ and $CoCl_2$ were found to be non-competitive inhibitors for AP-2 with Ki for NaF 0.29 mM, for $SnCl_2$ 1.67 mM and $CoCl_2$ 2.5 mM. Inhibition of AP-3 by $ZnCl_2$, NaF and $SnCl_2$ were also found to be non-competitive where Ki for $ZnCl_2$ was 0.27 mM, for NaF 0.30 mM and $SnCl_2$ 2.04 mM. NaF was a non-competitive inhibitor for all three forms of enzyme. Zn^{+2} ion caused mixed type of inhibition for AP-2, Co^{+2} ions

were non-competitive inhibitors for AP-2 but appeared competitive inhibitor for AP-1. PO_4^{-3} was found to be a competitive inhibitor for all forms of acid phosphatases (Table 4).

DISCUSSION

Acid phosphatases are mostly represented as multiple forms of molecular weights very close to one another and were very difficult to resolve as homogenous enzyme. Multiple acid phosphatases have been partially purified from different plant sources like leaves and nodules of *Phaseolus vulgaris* (Garcia *et al.* 2004), garlic seedlings (Yenigun and Guvenilir 2003), however single form of acid phosphatase was also found in different plants like developing pea seed coat (Murray 1980), germinating peanut seed (Gonnety *et al.* 2007), aquatic plant *Spirodela oligorrhiza* (Hoehamer *et al.* 2005), banana fruit (Turner and Plaxton 2001). Enzyme preparations (AP1, AP2 and AP3) purified in this study may represent mixtures of more than one enzyme, hence are more appropriately said to be partially purified acid phosphatases fractions.

All three fractions showed pH optima of 5.5 (Fig. 2A) similar to many plant acid phosphatases (Turner and Plaxton 2001, Garcia *et al.* 2004, Yenigun and Guvenilir 2003, Gonnety *et al.* 2006), though there are some reports of plant acid phosphatases having pH optima of 3 and 6 (Andriotis and Ross 2004). Similar to other plant acid phosphatases (Zheng and Duranti 1995, Turner and Plaxton 2001, Garcia *et al.* 2004, Gonnety *et al.* 2006, 2007), all three fractions of enzyme showed high thermal stability (Fig. 3) and temperature optima of 45-60°C (Fig. 2B). Plant acid phosphatases are mostly defined as non-specific as they act on a wide variety of substrates while alkaline phosphatases are reported to be specific for their substrates. AP1, AP2 and AP3 enzymatic preparations act on broad range of substrates (Fig. 4A) but they differ from one another in their order of preference for these substrates. For all three enzymatic preparations, PPi was the best substrate and phytic acid was the poorest substrate. 3-PGA was the preferred substrate for AP1 and AP3 but not for AP2. ATP and PPi were reported to be good substrates and sugar phosphates as poor substrates for acid phosphatases isolated from various plant sources (Turner and Plaxton 2001, Garcia *et al.* 2004, Andriotis and Ross 2004, Yoneyama *et al.* 2004, Gonnety *et al.* 2006, 2007). Reasons for showing high activity with PPi might be the involvement of these enzymes in energy metabolism as earlier stage of seed development can be a metabolically active stage. Among three natural substrates (3-PGA, PPi, FBP) taken for studying kinetic properties, Km for 3-PGA was found to be the lowest and Vmax was found highest with PPi for all three enzymatic fractions (Table 3). Km for PPi of acid phosphatases studied in different plants showed a wide range in cotyledons of soybean seeds (Ullah and Gibson 1988), 0.235 mM in embryonic axes of *Glycine max* (Islas-Flores and Villanueva 2007) and 1.03 mM in banana fruits (Turner and Plaxton 2001).

There are different reports for the effects of Ca^{+2}, Mg^{+2}, EDTA in literature, these can be activators (Turner and Plaxton 2001, Yenigun and Guvenilir 2003, Gonnety *et al.* 2007, Islas-Flores and Villanueva 2007),

Table 4. Ki values and kinds of inhibition of acid phosphatases by different inhibitors

Inhibitor	AP1		AP2		AP3	
	Ki(mM)	Type of inhibition	Ki(mM)	Type of inhibition	Ki(mM)	Type of inhibition
KH_2PO_4	0.35	CI	0.36	CI	0.17	CI
$ZnCl_2$	0.16	NCI	nd	MI	0.27	NCI
NaF	0.28	NCI	0.29	NCI	0.30	NCI
$CoCl_2$	4.29	CI	2.5	NCI	nd	nd
$SnCl_2$	1.47	NCI	1.67	NCI	2.04	NCI

CI, Competitive Inhibition; NCI, Non-Competitive Inhibition; MI, Mixed Inhibition; nd, inhibition not determined

inhibitors (Gonnety *et al.* 2007), without any effect (Murray 1980) on plant acid phosphatases. Enzymatic preparations in this study were not affected by Ca^{+2} and Mg^{+2} but activated by EDTA (Fig. 4B) though extent of activation varies for different enzyme preparations from 45% in AP1, 24% in AP2 and 12% in AP3. Hg^{+2} and molybdate ions were reported to be strong inhibitors of acid phosphatases in kidney bean, soybean, rice, banana fruit (Ferreira *et al.* 2000, Turner and Plaxton 2001, Garcia *et al.* 2004, Yoneyama *et al.* 2004) and were found to be the same for these enzyme preparations. Phosphate ions are reported as competitive inhibitors for acid phosphatases (Duff *et al.* 1994, Ferreira *et al.* 2000, Turner and Plaxton 2001, Andrioits and Ross 2004). Feedback inhibition of acid phosphatases by Pi may represent a general form of cellular regulation of these enzymes.

In summary, it can be concluded that acid phosphatases though widely studied in plants, the actual role played by them is still unclear. This group of enzymes share common features i.e. variability in their subunit structures and substrate specificities. Most of the time, multiple forms of acid phosphatases are reported in different plant parts (Duff *et al.* 1994, Garcia *et al.* 2004) which differ from one another in molecular weights and their substrate specificities. Moreover, variability in molecular weight and substrate specificity has also been reported to be changing during the development of root nodules (Gupta *et al.* 1998, Kaur *et al.* 1999) though in other plant parts, such type of data is mostly lacking. These various enzymatic forms might have arisen either by the appearance/disappearance of forms or by simply association/disassociation of subunits of enzyme. Studies focused on isolations and determination of features of different forms of these enzymes may lead to the elucidation of actual role played by them in plant metabolism. Acid phosphatases have also been reported to be involved in plant defense mechanism against abiotic/biotic stresses (Plaxton and Carswell 1999) and are induced under Pi-starvation (Bozzo *et al.* 2002).

ACKNOWLEDGEMENT

The co-author, V K Zhawar, is thankful to the Council of Scientific and Industrial Research, New Delhi for the award of Junior Research Fellowship.

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