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Purification and characterization of a heat-stable acid phosphatase from chickpea

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Acid phosphatases (APases) form a group of enzymes catalyzing hydrolysis of a variety of phosphate esters in the acidic environments. APases are believed to increase orthophosphate (Pi) availability under phosphorous deficient conditions. The APase from chickpea was purified by ethanol precipitation, followed by successive chromatographies on DEAE-Cellulose and Sephadex G-150. The enzyme was purified 73-folds. The optimum temperature and pH for enzyme activity were 50 °C and 5.5, respectively. All metal salts except ZnCl₂ and HgCl₂ were tolerated and did not adversely affect APase activity. The purified enzyme had a lower K_m (0.25 mM) and higher V_{max} (9 mM) for PNPP. The APase had broad substrate specificity with PNPP and natural substrates.

Key words: Acid Phosphatase activity, chickpea, purification

Acid phosphatases (APases) form a group of enzymes catalyzing hydrolysis of a variety of phosphate esters in the acidic environments. APases are believed to increase orthophosphate (Pi) availability under phosphorous deficient conditions (Vance *et al.*, 2003). In most agricultural soils, organic P comprises 30-80% of the total P (Dalal, 1978). The largest fraction of organic P, approx 50%, is in the form of phytin and its derivatives (Tarafdar and Claassen, 1988). For organic P sources in the soils to be used, they must be first hydrolyzed by phosphatases. Free soluble phosphate reserves play a vital role in energy transfer, metabolic regulation, important structural constituent of biomolecules like phytin bodies in the ungerminated seeds, protein and nucleotide phosphorylation (Finche, 1989; Ehsanpour

and Amini, 2003). Enhanced excretion of APases under P-deficient conditions has been documented in a number of plants (Tarafdar and Claassen, 1988; Vance *et al.* 2003). A positive relation was reported between root APases and phosphorous uptake in bean (Helal, 1990) and barley (Asmar *et al.*, 1995). A negative relationship was also observed between APases and phosphorous uptake under low phosphorous stress in wheat (Barret-Lennard *et al.*, 1982). Here in the present study we report details on purification and characterization of thermo stable acid phosphatase enzyme from chickpea. Chickpea is one of the most important crops in terms of its high nutritive value after soybean, peanut and common bean.

Materials and methods

Enzyme extraction and purification

The enzyme was extracted from the cotyledons of 7 days old seedlings. Briefly, the tissue was ground with mortar and pestle at 0-4°C using 100 mM sodium acetate buffer (pH 5.0). The homogenate was centrifuged at 8000g for 15 min, and the supernatant collected. All the steps for enzyme purification were done at 4°C. The crude enzyme was first concentrated by ethanol precipitation then applied on a DEAE-cellulose column (1 x 10 cm) pre-equilibrated with 100 mM sodium acetate buffer (pH 5.0). The adsorbed proteins were eluted in a salt gradient (0-1 M) at a flow rate of 25 ml/h. The fractions showing acid phosphatase activity were pooled and subjected to gel filtration chromatography using a column (50 x 1.0 cm) packed with sephadex G-150. The column was pre-equilibrated 100 mM sodium acetate buffer (pH 5.0). The adsorbed proteins were eluted in 100 mM sodium acetate buffer (pH 5.0). The separated and purified fractions were stored at -20 °C till further analysis. The enzyme activity was measured as described previously (Sharma *et al.*, 2007). One unit of enzyme activity was defined as the amount of enzyme that released one μ mole of product per minute from substrate at 37 °C.

Kinetic parameters

The Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) for p-nitro phenol phosphate (PNPP, 0.1-2mM), and Phosphoenolpyruvate (PEP, 0.1-2 mM) were determined from simple Lineweaver-Burk plots.

Effect of pH and temperature on enzyme activity

The effect of pH on enzyme activity was determined in pH range of 3-8, using 200 mM sodium acetate (pH 3.0-6.0), and Tris-Maleate (pH 6.0-8.0) buffers. The enzyme activity was measured at 37°C for 1 h. The effect of temperature on enzyme activity was

measured at pH 5.5 in 200 mM sodium acetate buffer over a temperature range of 30-70°C. Thermo stability in the absence of substrate was determined by measuring the residual activity of the enzyme at different intervals after incubation at 50 and 60°C temperatures.

Effect of metal ions on enzyme activity

The effect of different metal ions on enzyme activity was examined by incubating various metal salts with enzyme extract in 200 mM sodium acetate buffer (pH 5.5) at 37 °C for 1 h. The enzyme activities remaining after incubation were determined under assay conditions.

Results and Discussion

APases in plants are classes of enzymes that display considerable heterogeneity with regard to their kinetics and functions (Duff *et al.*, 1994). This complexity may contribute to conflicting reports regarding role of APA in phosphorous nutrition. APases are reported to be induced under phosphorous (P_i) deficiency, in order to maintain certain level of P_i inside the cells (Barrett-Lennard *et al.*, 1982; Olmos and Hellin, 1997). Earlier study in our lab reported on studies on acid phosphatase (APase) activities during various germination stages in chickpea (Kaur *et al.*, 2001). The present study reports on purification and characterization of acid phosphatase from chickpea. The enzyme was purified from the chickpea at 7 days after germination since maximum activity was observed at this stage (data not shown). The results of purification of APase from chickpea extract were obtained as shown in Table 1. The supernatant obtained from ethanol precipitates were first loaded on an ion-exchange column of DEAE-cellulose. The elution profile using a linear gradient of 1 M NaCl in DEAE-cellulose chromatography showed a broad activity peak (fraction No. 5-9) which was nearly coincided with protein peak (Fig. 1A). Further purification was

pursued by Sephadex G-150 gel permeation column (Fig. 1B). The active fractions eluted from DEAE-cellulose were pooled, concentrated and loaded onto the Sephadex

G-150 gel permeation column which resulted in 73-fold purification as compared to the crude extract.

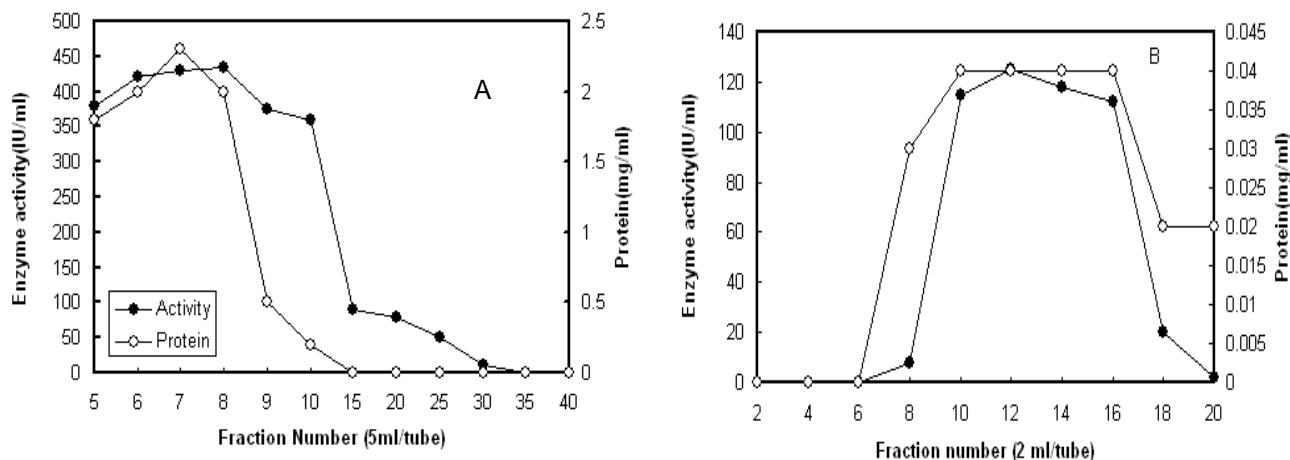


Fig. 1: Elution profile of DEAE-Cellulose (A) and Sephadex G-150 (B) column chromatographies for APase purification

Kinetic parameters of purified APase

Besides hydrolyzing PNPP, the APase also hydrolyzed PEP, with almost equal K_m values 0.25 and 0.22, respectively (Table 2). The values obtained were substantially lower than 0.7 and 0.4 of APase obtained from *Vigna radiate* (Kundu and Banerjee, 1990). Although the purified APase showed broad substrate specificity, but higher V_{max} (9.09 IU/mg) was observed with PNPP.

Table 1: Partial purification of acid phosphatase

Steps of Purification	Total activity Units (IU)	Total Protein (mg)	Specific activity (IU/mg)	Purification Fold
Crude	28000	525	53	1.0
Ethanol precipitation	11250	75	150	2.8
DEAE Cellulose	435	2.2	197	3.7
Sephadex G-150	125	0.032	3906	73.2

Table 2: Substrate specificity of acid phosphatase

Substrate	K_m (mM)	V_{max} (IU/mg)
PNPP	0.25	9.09
PEP	0.22	4.76

Table 3: Effect of pH on activity of acid phosphatase

pH	% Activity
3.0	9
3.5	18
4.0	31
4.5	54
5.0	80
5.5	100
6.0	77
6.5	63
7.0	54
7.5	34
8.0	23

Values are mean of duplicate assays
Maximum activity has been considered as 100

Table 4: Effect of Temperature on activity of acid phosphatase

Temperature (°C)	% Activity
30	46
35	52
40	62
45	70
50	100
55	85
60	60
65	28
70	8

Table 5: Thermal stability of acid phosphatase

Incubation Time (min)	Temperature (°C)	
	50°C	60°C
% Activity		
0	100	100
30	91	64
60	83	51
120	58	42

Table 6: Substrate specificity of acid phosphatase

Substrate (2 mM)	Enzyme activity (IU)
PNPP	30
α -Naphthyl acid P	1
ATP	2.5
PEP	1.6
F1,6 BP	0.85
G-1-P	0.76
G-6-P	0.54
F-6-P	0.33
Phytic acid	0.22

Effect of pH and temperature on enzyme activity

The activity of purified enzyme was measured at various pH values ranging between 3.0 and 8.0. As shown in Table 3, maximum activity was obtained at pH 5.5.

The pH optimum of 5.5 is in agreement with the general range of many microbial sources reported so far: *Vigna radiate* (Kundu and Banerjee, 1990), soybean (Kaneko *et al.*, 1989), wheat (Verzee, 1969) and pea (Murray and Colliar, 1977). To determine the optimum temperature for enzyme activity, reactions were performed at various temperatures (30-70 °C) at pH 5.5 for 1 h. APase showed maximum activity at 50 °C (Table 4). Optimum of 50°C observed was higher than 45 reported for Japanese radish (Yoshimoto *et al.*, 1992). For determining the thermo stability, the enzyme solution was heated at 50 °C and 60 °C for up to 120 min at pH 5.5. After treatment, the residual activities were measured at 50 °C and 60 °C (Table 5). The APase retained about 60% and 40% activity after 120 h at 50 °C and 60 °C, respectively. The thermo stability of APase was similar to lupin Apase (Zheng and Duranti, 1995) and Apase from *Vigna sinensis* (Biswas and Cundiff, 1991), which have been reported as the most thermostable APase's and maintain about 60% activity, respectively, after 60 of incubation at 60 °C. So overall, this lower pH and high temperature optima may offer advantageous to combat temperature changes in the field conditions during Pi assimilation from soil.

Substrate specificity of acid phosphatase

APase showed maximum activity with PNPP (Table 6). For natural substrates, activity was maximum with ATP. In general APase purified was able to hydrolyze variety of substrates and have relatively broader substrate specificity.

Effect of salts and other reagents on enzyme activity

The effect of various salts on APase activity was investigated at a concentration of 2 mM (Table 7). The Hg²⁺, which is known to affect -SH-groups, completely abolished the APase activity. The strong inhibitory effect

observed with Hg²⁺ suggested that some -SH- group in the protein might be essential for the activity. Only CoCl₂ positively modulated APase activity, whereas the modulatory effect of CaCl₂ was marginal.

Table 7: Effect of metal salts on activity of acid phosphatase

Compound	Relative activity (%)
Control	100
Sodium molybdate	53
ZnCl ₂	59
NaCl	100
MgCl ₂	100
EDTA	117
CaCl ₂	117
HgCl ₂	23
CoCl ₂	132
NaH ₂ PO ₄	62

In conclusion, we have reported the purification of a novel heat-stable APase from chickpea. The temperature and pH optima of 50°C and 5.5, respectively, coupled with its thermostable nature, make this APase a potential candidate for plant breeding studies. Cloning and immobilization of this thermostable enzyme is also underway for exploiting its commercial potential.

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