Research in Plant Biology, 1(4):01-06, 2011 ISSN : 2231-5101
www.resplantbiol.com

Regular Article

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

Parminder Kaur, Arun Dev Sharma* and Gurmeen Rakhra

PG Department of Biotechnology, Lyallpur Khalsa College, G T Road, Jallandhar-144001, Punjab, India
*Corresponding e-mail: arundevsharma@hotmail.com, arundevsharma47@rediffmail.com

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 $ZnCl_2$ and $HgCl_2$ 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 activiy, 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 8000,g 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 µmole 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 (Pi) deficiency, in order to maintain certain level of Pi 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.

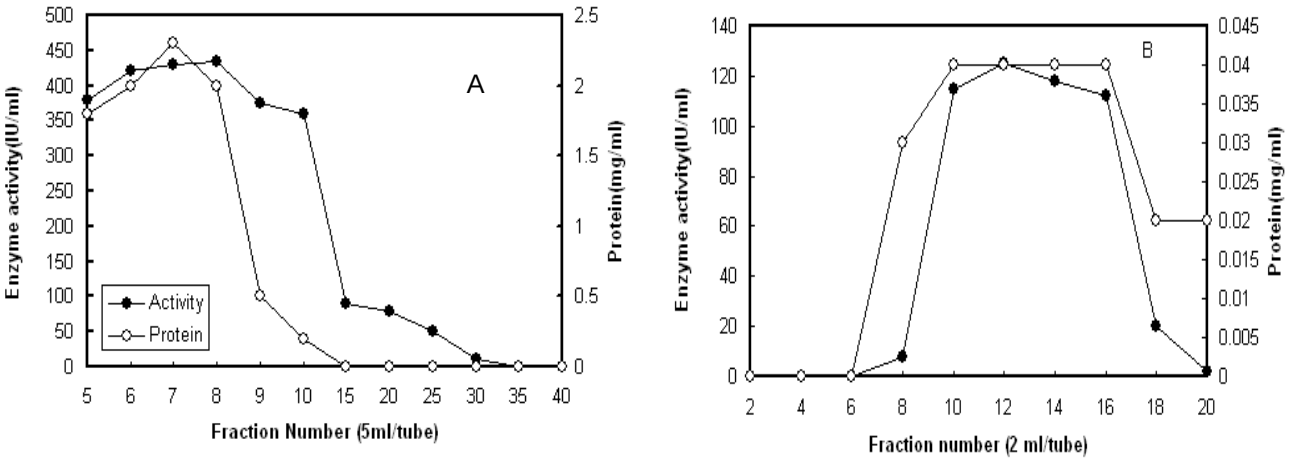

Fig. 1: Elution profile of DEAE-Cellulose (A) and Sepahdex G-150 (B) column chromatographies for APase purification

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)	Purifi-cation 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 sinesis* (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 Hg^{2+}, which is known to affect -SH-groups, completely abolished the APase activity. The strong inhibitory effect

observed with Hg^{2+} suggested that some –SH- group in the protein might be essential for the activity. Only $CoCl_2$ 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 molybedate	53
ZnCl2	59
NaCl	100
MgCl2	100
EDTA	117
$CaCl_2$	117
$HgCl_2$	23
$CoCl_2$	132
NaH_2PO_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 immobilize-ation of this thermostable enzyme is also underway for exploiting its commercial potential.

Acknowledgement

AD Sharma would like to thank DST, Govt. of India for providing financial assistance for the present study.

References

Asmar, F., T. Gahoonia, N. Nielsen, 1995. Barley genotypes differ in activity of soluble extra cellular phosphates and depletion of organic phosphorous in the rhizosphere soil. Plant Soil, 172, 117-122.

Barrett-Lennard, E.D., A.D. Robson, H. Greenway, 1982. Effect of phosphorus deficiency and water deficit on phosphatase activities from wheat leaves. J. Expt. Bot., 33, 682-693.

Biswas, T.K., C. Cundiff, 1991. Multiple forms of acid phosphatase in germinating seeds of *Vigna* sinesis. Photochemistry, 30, 2119-2126.

Bohnert H.J., D.E. Nelson, R.G. Jensen, 1995. Adaptations to environmental stresses. Plant Cell, 7, 1099-1111.

Dalal, R.C. 1978. Organic phosphorous. Advances in agronomy 29, 83-117.

Duff, S.M.G., G. Sarath, W.C. Plaxton, 1994. The role of acid phosphatase in plant phosphorus metabolism. Physiol. Plantarum, 90, 791-800.

Ehsanpour, A.A., F. Amini, 2003. Effect of salt and drought stresses on acid phosphatase activities in alfalfa (*Medicago sativa* L.) explants under in vitro culture. Afr. J. Biotechnol., 2,133-135.

Elliot, G.C., A. Lauchli, 1986. Evaluation of an acid phosphatase assay for detection of phosphorus deficiency in leaves of maize (*Zea mays* L.). J. Plant Nutr., 9, 1469-1477.

Fernandez, D.S., J. Ascencio, 1994. Acid phosphatase activity in bean and cowpea plants grown under phosphorus stress. J. Plant Nutr., 17, 229-241.

Fincher, G.B. 1989. Molecular and cellular biology association with endosperm mobilization in germinating cereal grains. Annual Rev. Plant Physiol. Plant Mol. Biol. 40: 305-346.

Furlani, A.M.C., R.B. Clark, J.W. Maranville, W.M. Ross, 1984. Root phosphatase activity of sorghum genotypes grown with organic and inorganic sources of phosphorus. J. Plant Nutr., 7, 1583-1595.

Helal, H.M., 1990. Varietal differences in root phosphatase activity as related to the utilization of organic phosphates. Plant and Soil, 123, 161-163.

Kaneko, J. M. Kuroiwa, K. Aoki, S. Okuda, Y. Kamio, K. Izaki, 1989. Purification and properties of acid phosphatases from axes and cotyledons of germinating soybeans. Agric. Biol. Chem., 54, 745-751.

Kaur, P., N Kaur A. K. Gupta, 2001. Pattern of acid phosphatase activity during chickpea (*Cicer arietinum*) seedling growth. In : Biochemistry - Environment and Agriculture. APS Mann, SK Munshi and AK Gupta (ed) pp393-97. Kalyani Publications, New Delhi, India.

Kundu, P.D., A.C. Banerjee, 1990. Multiple forms of acid phosphatase from seedlings axes of *Vigna radiate*. Phtochem., 29, 2825-2828.

Mclachlan, K.D., D.G. Demarco, 1982. Acid phosphatase activity of intact roots and phosphorus nutrition in plants: III. Its relation to phosphorus garnering by wheat and a comparison with leaf activity as a measure of phosphorus status. Aust. J. Agric. Res., 33, 1-11.

Murray, D.R., M.D. Colliar, 1977. Acid phosphatase activities in developing seeds of *Pisum sativum*. Aus. J. Plant Physiol.

Olmos, E., E. Hellin, 1997. Cytochemical localization of ATPase plasma membrane and acid phosphatase by cerium based in a salt-adapted cell line of *Pisum sativum*. J. Exp. Bot., 48, 1529-1535.

Sharma A. D., R. Kaur, 2007. Drought-induced changes in acid phosphatase activities in wheat in relationship with phosphorus. Emiratus Journal of Agricultural Sciences, 19: 31-38.

Shinozaki, K., K. Yamaguchi-Shinozaki, 1996. Molecular responses to drought and cold stress. Curr. Opinion In Biotechnol., 7, 161-167.

Szabo-Nagy, A.G., G. Galiba, E. Erdei, 1992. Induction of soluble phosphatases under ionic and non-ionic osmotic stress in wheat. J. Plant Physiol., 140, 329-633.

Tarafdar, J.C., N. Claassen, 1988. Organic phosphorous compounds as a phosphorous source of higher plants through the activity of phosphatases produced by plants roots and microorganisms. Biol.Fertility of Soils, 5, 308-312.

Tsvetkova, G.E., G.I. Georgiev, 2003. Effects of phosphorous nutrition on the nodulation, nitrogen fixation and nutrient use efficiency of *Bradyrhizobium japonicum*-soybean (*Glycine max* L. Merr.) symbiosis. Bulg. J. Plant Physiol., special issue, 331-335.

Vance, C.P., C. Uhde-Stone, D.L. Allan, 2003. Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New Phytol., 157, 423-447.

Verzee, ZHM. 1969. Isolation of three acid phosphatases from wheat germ. Eur. J. Biochem. 9, 439-444.

Yan, X., H Liao, C.T, Melanie, E.B, Steve, J.P. Lynch, 2001. Induction of a major leaf acid phosphatase does not confer adaptation to low phosphorous availability in common bean. Plant Physiol., 125, 1901-1911.

Yoshimoto, M., T. Kimura, T. Miyamoto, J. Sakamoto, S. Hatano, 1992. Purification and properties of acid phosphatase from Japanese radish. Bioscience Biotechnol. Biochem. 56, 147-148.

Zheng, Y., M. Durani, 1995. Molecular properties and thermal secretion of lupin seed phosphatases. Photochemistry 40, 21-22.