PARTIAL PURIFICATION,CHARACTERIZATION AND SOME KINETIC PROPERTIES OF LOW MOLECULAR WEIGHT ACID PHOSPHATASE FROM LEAVES OF GERMINATING *VIGNA RADIATA* SEEDS.

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ABSTRACT

Acid phosphatase isoenzyme (AcP-II) from leaves of germinating seeds of *vigna radiata* (mung beans) was partially purified by CM-Cellulose chromatoghraphy, gelfiltration on Ultrogel AcA 44 and Con A-Sepharose affinity chromatoghraphy. The specific activity of 25U/ mg of protein was obtained with recovery of 4 %. The enzyme showed a purification by a factor of 86. Gel filtration experiment and sodium dodecyl sulphate polyacrylamide gel electrophoresis indicated that the isoenzyme had a molecular weight of 29 kDa. The K_m value of the isoenzyme was 0.5 mM with p-nitrophenyl phosphate as substrate. The enzyme had pH optimum of 5.5 and optimum temperature of 60^{o}C. The enzyme was inhibited by phosphate, vanadate, fluoride and molybdate. It was also strongly inhibited by Cu^{++}, Hg^{++}, Zn^{++} and Al^{+++}. The enzyme had very little effect of inhibition by thiol specific reagents, such as iodoacetamide, N-ethyl-maleimide etc., suggesting that no -SH groups are involved in the enzyme catalysis. Dithiothreitol and β-mercaptoethanol had small activating effect at low concentrations indicating their properties as reducing agents but at their high concentrations, the activation was replaced by inhibition, suggesting that these thiols may cause a conformational change in the enzyme at a place other than the active site. Variation of K_m values with pH alteration study showed that a histidine may constitute a part of an active site. This was confirmed by inhibitory effect of high concentration of iodoacetate at pH 7.2

Key words: Acid phosphatase; Mung beans; Vigna radiata; purification; characterization.

INTRODUCTION

Acid phosphatases (EC 3.1.3.2) are enzymes that catalyze the removal of inorganic phosphate from phosphomonoesters in acid media (Anand and Srivastava, 2012). These are ubiquitous in nature and found in bacteria, fungi, animals and plants (Guo and Pesacreta,1997; Leitao *et al.,* 2010; Naz *et al.,* 2006; Siddiqua *et al.,* 2008; Al-Omair, 2010). The studying acid phosphatases is difficult due to their occurrence in multiple forms and their small quantity (Park and Van Etten, 1986; Waymack and Van Etten, 1991). Their study is even more difficult because of wide variations in the activity and variations of multiple forms between species and between different organs during various stages of plant development (Alves *et al.,* 1994; Baes and Van Cutasem, 1993). Four isoforms in *v. sinensis* (Biswas and Cundiff, 1991) and six isoforms in *v.* mungo (Haraguchi *et al.,* 1990), rise in activity in the axes during the germinating of soybean seeds at the early stage (Okuda *et al.,* 1987) and also the increase of acid phosphatase activity of 10-fold in soybean leaves on seed pod removal after flowering season (Staswick *et al.*,1994) are the examples of the facts. These factors together with occurrence of their small quantity and instability in dilute solution, makes the isolation of highly purified acid phosphatase difficult.

A number of acid phosphatases have been purified to homogeneity or near to homogeneity from the different plant sources such as sweet potato (Durmus *et al.*,1999), aleurone particles of rice grain (Yamagata *et al.,* 1980), cotton seed (Bhargava and Sachar,1987), lupin seed (Olczak *et al.,* 1997), cotyledons of germinating soybean seeds (Ullah and Gibson, 1988), axes and cotyledons of germinating soybeans (Kaneko *et al.,* 1990) in order to study their structures and functions in the cells. The physiological roles of acid phosphatase are not well understood because of the heterogeneity and lack of substrate specificity (Duff *et al.,* 1994). Generally, acid phosphatases are believed to function in the production, transport and recycling of inorganic phosphate (Pi) (Yoneyama *et al.,* 2004). In plant roots, acid phosphatases seem to be involved in the solubilization of macromolecular organic phosphates in soils by its catalytic action to release Pi which can then be absorbed by plants (Panara *et al.,* 1990) for growth and development. In tubers, Kouadio (2004) described the important role acid phosphatase concerning the transport

of Pi in metabolic processes during the preservation of cocoyam. In seeds and seedlings, the physiological function of the acid phosphatase is to provide inorganic phosphate to the growing plant during the germination and many different phosphate esters of sugars and phosphorylated compounds stored in seeds and seedlings are hydrolyzed to release Pi through the catalytic action of increased enzyme activity which is either due to *de novo* synthesis of enzyme protein or activation of the enzyme by imbibition (Gahan and Mc Lean, 1969; Schultz and Jensen,1981; Akiyama and Suzuki, 1981; Hoehamer, et al., 2005).

Most of the purified plant acid phosphatases have been found to contain molecular weights from 50 kDa to 200 kDa (Gonnety *et al.*, 2006) and very few reports on low molecular weight acid phosphatases (18 kDa -31 kDa) have been cited in the literature.

In our preceding report (Nadir *et al.*, 2012), we attempted to purify 29 kDa acid phosphatase to homogeneity from germinating *v. radiata* seeds (whole plants, axes and cotyledons) and characterized with respect to molecular weight, pH optimum, K_m, V_{max}, and K_i values with various inhibitors etc., This paper describes the presence of multiple forms of acid phosphatase in the leaves of germinating *v. radiata* seeds and presents a simple procedure of purification of low molecular weight form of acid phosphatase along with some kinetic properties. This kinetic data provides a basic knowledge about the structure at the active site.

MATERIAL AND METHODS

Chemicals: Ion-exchanger CM-Cellulose from Whatman Biosystem, gel media Sephadex G-100 and Ultrogel AcA 44, affinity gel Concanavalin A-Sepharose 4B (Con A-Sepharose) and marker proteins were obtained from Sigma Chemical Chemical Co. The chemicals for SDS-PAGE were supplied by Sigma-Aldrich Chemical Co. Substrates and other chemicals were purchased from Fluka and BDH Chemical Company.

Enzyme assays: Acid phosphatase activity was determined as described by Panara *et al.* (1990). To 900µl solution of substrate containing 4mM p-nitrophenyl phosphate in 0.1M acetate buffer pH 5.5, 100µl of enzyme solution was added and incubated at 37°C for 5 min. The reaction was terminated by adding 1-4 ml of 0.1N KOH and the intensity of the yellow color (phenolate ions) produced was measured at 405nm (ε = 18000 M^{-1} cm^{-1}). One unit of enzyme was defined as amount of the enzyme that produces 1µ mol of p-nitrophenol / min. Specific activity was expressed as enzyme units / mg of proteins.

The pH dependence of enzyme activity was determined by measuring the hydrolysis of p-nitrophenyl phosphate at 37°C in a series of buffer at various pH values ranging from 3.6 to 9.0. Buffers used were 0.1 M sodium acetate buffer from pH 3.1 to 6.0 and Tris-HCl buffer (0.1M) from pH 7.0 to 9.0.

The temperature optimum was determined by measuring the activities at temperatures between 40-80°C at intervals of 5°C. To determine the temperature stability, the enzyme was first pre-incubated in 0.1 M acetate buffer, pH 5.5 at different temperatures ranging from 50°C to 80°C for 30 min. Following cooling at 4°C, the enzyme assay was done as usual to estimate residual activity.

Substrate specificity studies were carried out by determining the release of inorganic phosphate as the result of hydrolysis of various substrates. Inorganic phosphate was determined by Black and Jones method (1983). The incubation mixture consisted of 450 µl of 0.1 M acetate buffer, pH 5.5 containing 4 mM of substrate and 50 µl enzyme solution, was incubated at 37° C for 5 minutes to release Pi from enzymatic reaction. This reaction was stopped by addition of 200 µl of 10 % trichloroacetic acid. The blue color was developed with molybdic acid reaction which was as follows: The 500 µl mixture (composed of 200 µl of 2 % ammonium molybdate and 300 µl of 14 % ascorbic acid in 50 % trichloroacetic acid) was added to the above mixture (700 µl) followed by the addition of 1 ml solution containing 2 % trisodium citrate and 2 % sodium arsinate in 2 % acetic acid to make the total volume of 2200 µl. The color was developed for 30 minutes and absorption was determined at 700 nm. The enzyme activity was expressed as a percent of p-nitro phenyl phosphate.

Inhibitions by metal ions: The mixture, consisting of 100 µl of the 0.1M cation solution under test, 600 µl of 1M acetate buffer pH 5.5 and 100 µl of enzyme solution, was pre-incubated for 10 min at 37°C. After pre-incubation, 200 µl of 20 mM p-nitrophenyl phosphate was added to determine the activity as usual. Simultaneously control and blank experiments were run in which the cation and enzyme solutions were replaced by water, respectively, in the pre-incubation mixtures.

Similarly, the effect of some compounds reacting with SH-groups of the enzyme on the enzyme activity at pH values varying from 3 to 9 was determined as described above.

Kinetic parameters: The K_m, V_{max} and K_i values were determined using p-nitrophenyl phosphate as the substrate in concentrations of 0.06 - 4 mM in the absence or presence of two or three fixed concentrations of inhibitors. These kinetic parameters were determined from Line-weaver-Burk plots. Straight lines were drawn by applying least square rule.

The pH dependence studies of K_m ,V_{max} and specificity constants were carried out as described by Pasqualini *et al.*(1997).

Protein determination: Protein concentration was determined by the Biuret method. In chromatographic procedures, the relative protein concentration was estimated from the absorbance at 280 nm.

Electrophoresis: SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli, (1970) under reduced conditions. The sample was prepared in sample buffer with β-mercaptoethanol and heated at 95°C for 2-3 min. The enzyme purity was checked in 12% acrylamide mini-slab gel. After the run, the proteins in gel were stained with coomassie blue and molecular weight estimates were made using standard size marker proteins as indicated in the respective figure.

Molecular weight determination: Two different acid phosphatase isoenzymes were placed on Ultrogel AcA 44 column (1.8x85 cm) separately and eluted with 0.01 M Tris-HCl buffer, pH 7.0 containing 0.1M NaCl. The operating flow rate was 25 ml/h and 5 ml fractions were monitored. The molecular weights of the isoenzymes were estimated on calibrated Ultrogel AcA 44 column by comparing its elution volumes with those of standard protein markers.

Germination of seeds, extraction and purification of enzyme: Seeds of *v. radiata* (mung beans) were washed with water three times and soaked in water for 3-4 h. After hydration, germination of seeds was performed on moist sand trays during at least 7 days in a room at ambient temperature of 28-35°C. The enzyme was purified by a procedure of Nadir *et al.* (2012) with slight modifications. The leaves obtained after 4-7th day of germinating seeds, were homogenized in Warring Blender with five volumes of 0.1M acetate buffer, pH 5.5. The homogenate was centrifuged at 45,000xg for30 min and supernatant was collected. To the supernatant, solid ammonium sulfate was added and brought to 80 % saturation. The mixture was stirred well and centrifuged. The precipitate obtained, was dissolved in 0.01 M acetate buffer, pH 5.9 and dialyzed against the same buffer over night. After centrifugation, the clear dialysate was applied to CM-Cellulose column (2.2x17.5 cm). The column was washed with buffer and some of the acid phosphatase activity peak (P-1) was eluted as unbound protein. After extensive washing, the bound acid phosphatase activity peak (P-II) was eluted by linear gradient of 0-0.5M NaCl in the same buffer (50ml) with flow rate of 40 ml/h. Fractions of approximate 10ml each were collected (Fig.1A). The enzymes from both peaks (P-1 and P-II) were pooled separately and concentrated to 5 ml each by ultrafiltration using Amicon YM2 membrane at 20 psi pressure. The enzyme sample, P-II was placed on Ultrigel AcA 44 column (1.8x85 cm) previously equilibrated and eluted with 0.05 M Tris-HCl buffer, pH 7.0 containing 0.1M NaCl. Fractions of 5ml were collected with flow rate of 25 ml/h (Fig. 1B). The highest activity containing fractions were pooled and dialysed against 0.01 M Tris-HCl buffer, pH 7.0 containing 1mM $Ca^{++}$and 1mM Mn^{++}. The dialysed enzyme was applied to a Con A-Sepharose column (2x11cm) which had been previously equilibrated with dialyzing buffer and washed with same buffer. The column was then eluted with 10 % α (+) D-methylglucopyranside in buffer. Fractions of 5 ml were collected at flow rate of 25 ml/h (Fig.1C). The most active fractions were pooled, concentrated by ultrafiltration and used for further study.

RESULTS AND DISCUSSION

Enzyme purification: Acid phosphatase isoenzyme (AcP-II) from leaves of germinating *v. radiata* seeds was partially purified. A summary of the purification is presented in table 1. About 86- fold purification was achieved with a specific activity of 25 U/mg of protein and a recovery of 4 %. The specific activity was higher in comparison with acid phosphatase purified from *Phaseolus vulgaris* to specific activity of 18.1 U/ mg of protein (Cabello-Diaz et al., 2012). However, it was much lower than the specific activity (598 U/ mg of protein) reported for acid phosphatase from *Euphorbia* latex (Pintus et al., 2011). The isoenzyme AcP-I could not be purified. The SDS-PAGE of AcP-II showed a major band with molecular weight at 29 kDa (Fig. 2). Very faint bands corresponding to 18 kDa and 14 kDa were also observed. These two bands are probably NH_2 – terminus truncated fragments originating from proteolysis during purification.

Gel filtrations of enzymes P-I and P-II obtained from CM-Cellulose chromatography on Ultrogel AcA 44 column have shown their elution profiles in figs.3A and 1B. The elution volumes of 100 ml and 115 ml for the peaks, AcP-1 and AcP-II were obtained. The molecular weights of these two native isoenzymes were found to be 58 kDa and 29 kDa (Fig. 3B) indicating that AcP-II is a monomeric protein. The 58 kDa acid phosphatase in *v. aconitifolia* seeds was also reported (Anand and Srivastava, 2013). These results showed the presence of multiple forms of acid phosphatase as reported in other plant sources (Panara *et al., 1*990; Pasqualini *et al.,*1997). Our results were consistent with acid phsphatases isolated from germinating blackgram (*v. mungo*)(Asaduzzman *et al.,* 2011) and wheat seedlings (Chen and Tao, 1989) which had molecular weights of 25 kDa and 35 kDa, respectively.

Effect of pH and temperature: Acid phosphatases isolated from leaves of *v. radiata* showed a pH optimum of 5.5 (Fig. 4). The same results were obtained for enzymes from seedlings of *v. radiata* (Nadir et al., 2012), caster bean seeds (Granjeiro *et al.,* 1999) and leaf of *p. vulgaris* (Tejera-Garcia *et al.,* 2004). But this optimum

pH value was higher than optimum pH (pH 4.75) of acid phosphatase from *Agaricus bisporus* (Wannet *et al.*, 2000).

The enzyme had optimum temperature of 60°C (Fig. 5) which was almost similar to the values reported for acid phosphatases purified from germinating soybean seeds (Ullah and Gibson, 1988) and axes of *v. radiata* seedlings (Kundu and Banerjee,1990). Optimum temperature of 60°C was higher than for barley roots (35°C) (Panara *et al.*,1990), cotton seeds and *zea mays* seeds (37°C), (Bhargava and Sacchar,1987; Senna *et al*., 2006), caster beans seeds (45°C)(Granjeiro *et al.*, 1999) and garlic seedlings (50°C)(Yenigun and Guvenilir, 2003). But was lower than optimum temperature (80°C) for isoenzymes from soybean seeds (Ferreira *et al.*, 1998).

The enzyme was found to be stable at 50°C. The same value was reported for the enzyme purified from seedlings of *v. radiata* (Nadir *et al.*, 2012), but it had lost 14 % of its activity at 55°C and 50 % of activity at 60°C after pre-incubation for 30 min. The enzyme was inactivated completely at 70°C. The enzyme from caster bean seeds was also stable and retained about 80% activity when pre-incubated for 30 min at 50°C (Granjeiro *et al.*, 1999). Similarly, the enzyme from soybean seeds was also found stable and retained 95-97 % activity at 60°C for 30 min (Ferreira *et al.*, 1998).

The relative hydrolysis rates of different phosphate esters are shown in table 2. The p-nitrophenyl phosphate, phenyl phosphate, β-naphthyl phosphate, phosphotyrosine and ATP were found good substrates while other substrates e.g., phosphoamino acids, sugar phosphates and nucleotides were hydrolyzed at slower rates. This broad range of substrate specificity was similar to those of other plant acid phosphatases (Tejera-Garcia *et al.*, 2004; Turner and Plaxton, 2001; Koffi *et al.*, 2010). A non-specificity of this enzyme seems to exhibit the metabolic significance in utilizing extracellular as well as intracellular phosphorylated compounds in release of Pi.

Action of modifiers and inhibitors: The action of various compounds as possible activators or inhibitors of acid phosphatase was determined. Alcohols such as methanol, ethanol and glycerol at concentrations of 10 % showed no activating effect on the activity, suggesting that acid phosphatase was not involved in the transphosphorylation reaction. The lack of effect of 4mM EDTA showed that divalent ions were not necessary for the activity. 1 % Triton X-100 activated the enzyme to 142 %. This activation may be due to its interaction with hydrophobic portions of the enzyme. Shekar *et al.*, (2002) had also reported the same effect on acid phosphatase from developing pea nut cotyledons. Two known acid phosphatase inhibitors, tartrate and citrate at concentration of 5 mM did not inhibit this enzyme. Tartrate resistance was also observed in many other acid phosphatases (Olczak *et al.*, 1997; Pan, 1987; Rossi *et al.*, 1981; Ching *et al.*, 1987; Doi, *et al.*, 1987). Phosphate, fluoride, vanadate and molybdate inhibited the enzyme as was the case for other phosphatases (Tabaldi *et al.*, 2008) and their inhibition pattern seemed to be very similar to our enzyme already reported (Nadir *et al.*, 2012). The comparison of their K_i values is shown in table 3. Phosphate acted as competitive inhibitor. Competitive inhibition was observed for acid phosphatase from *artemecia vulgaris* pollen extract (Cirkovic *et al.*, 2002) and from other plant sources. Vanadate was also competitive inhibitor of this enzyme. This result was in accord with the findings of purple acid phosphatase in the walls of tobacco cells (Kaida *et al.*, 2008). Fluoride inhibited non-competitively. A similar type of inhibition was reported for acid phosphatase from rice plants (Tso and Chen, 1997) whereas the molybadate showed a very strong inhibition of mixed type (K_i 3 µM). Such type of inhibition was obtained in enzymes from axes and cotyledons of germinating soybeans (Kaneko *et al.*, 1990).

Effect of metal ions: Metal ions showed different effects on the acid phosphatase activity. The activity was reduced by Fe^{++}, Cu^{++}, Hg^{++} and Zn^{++} which was consistent with the results reported (Tso and Chen, 1997; Bozzo *et al.*, 2004), while other divalent ions such as Ca^{++}, Mg^{++} and Mn^{++} had no significant effect on activity. The Zn^{++} and Hg^{++} inhibited the enzyme non-competitively with K_i values of 4 mM and 13 µM, respectively (Table 3). It was observed from the Lineweaver-Burk plot while calculating the kinetic parameters, both K_m and V_{max} decreased with elevation in Hg^{++} concentration. The data demonstrated that $HgCl_2$ concentrations ranging from 0.01-0.02 mM decreased the apparent K_m values from 28 % to 37 % of real K_m value while the enzymatic activity, apparent V_{max} values decreased from 40 % to 57 % of real V_{max}. By using a Cornish-Bowden plot, the K_i was found to be 13µM (Table 3). The effects of EDTA and β-mercaptoethanol on the inhibition of enzyme activity by some metal ions are shown in Table 4. At 12.5 mM concentrations, Al^{+++}, Zn^{++} and Hg^{++} showed around 80 % 78 % and 100 % inhibitions, respectively (Table 4a and b). The addition of 20 mM EDTA to portions of enzyme solutions which had been inhibited by metal ions showed that inhibition was substantially reversed. The activity recovery was 54 %, 66 % and 12 %, respectively (Table 4a). But addition of β-mercaptoethanol at 10 mM concentration was found without effect on the inhibition by these metal ions. By this treatment enzyme activity recovery was 0 % (Table 4b). In general, inhibition caused by these oxidizing agents (metal ions) was not reversed by reducing agent (β-mercaptoethanol).

Effect of some SH-reacting compounds: Pre-incubating the enzyme with SH-reacting compounds such as iodoacetic acid (5mM), iodoacetamide (10mM), N-ethyl-maleimide (10mM) and p-hydroxymercuri-benzoate (0.5mM) for 10 min at various pH ranging from 3 to 8, before the addition of p-nitrophenyl phosphate showed that these compounds inhibited the enzyme to lesser extent (10-20%) revealing that SH-group containing amino acids in the enzyme may not be significant for its catalytic activity. The suitable controls indicated that enzyme was almost stable over pH range 3-8 (Table 5). Similar to our observation, Panara *et al*. (1990) found no substantial role of free SH- groups in the barley root acid phosphatase but Granjeiro *et al*.(2003) demonstrated the importance of SH- groups in the catalytic mechanism of caster bean seed acid phosphatase.

Effect of some SH-protecting compounds: Under above stated conditions, the pre-incubation of enzyme with dithiothreitol (DTT) or β-mercaptoethanol at 12.5 mM for 10 min at pH 5.5 showed activation by nearly 5 %. The effect of these two compounds on the enzyme activity was further studied at their different concentrations when added to the enzyme at the same time as the p-nitro phenyl phosphate solution at pH 5.5. The results are shown in Table 6. A small activation (6-10%) was observed with concentrations up to 100 mM of DTT while β-mercaptoethanol at these concentrations displayed very little or no activation. Thus, DTT or β-mercaptoethanol at low concentrations behaves as reducing agent. At high concentrations of DTT or β-mercaptoethanol (200-500 mM), activation was replaced by inhibition (Table 6). Prolonged incubation of enzyme with 50 mM of DTT or β-mercaptoethanol at 4^{o}C, a very little activation was observed but ascorbic acid caused strong inhibition and complete inhibition was obtained in 17 days (Table 7). Thus DTT or β-mercaptoethanol has a stabilizing and protective effect on the enzyme activity.

From the above discussion, it may be concluded that this enzyme was not susceptible to inactivation by some SH- protecting reagents (DTT, β-mercaptoethanol or ascorbic acid) or SH-blocking reagents (iodoacetic acid, iodoacetamide, N-ethyl-maleimide or p-hydroxymercuri-benzoate) and thus the SH-groups seemed to have no catalytic role in the mechanism of enzyme action. The inhibition by DTT or β-mercaptoethanol at very high concentrations may cause influence on the catalytic process perhaps by producing conformational changes in regions other than the active site.

The effect of pH on the K_m and V_{max} of acid phosphatase is shown in Table 8. At lower pH values there seemed to be a trend of decrease in V_{max} but K_m was almost the same at each pH. It may be suggested that some ionizable groups were protonated, resulting in a slow rate of hydrolysis. The protonation of ionizable group was not affecting the substrate affinity for the enzyme as the K_m values in range of pH 4.6-5.8 were the same. The similar finding was also reported by Anand and Srivastava (2013), confirming that this ionizable group may not be located in the enzyme active site.

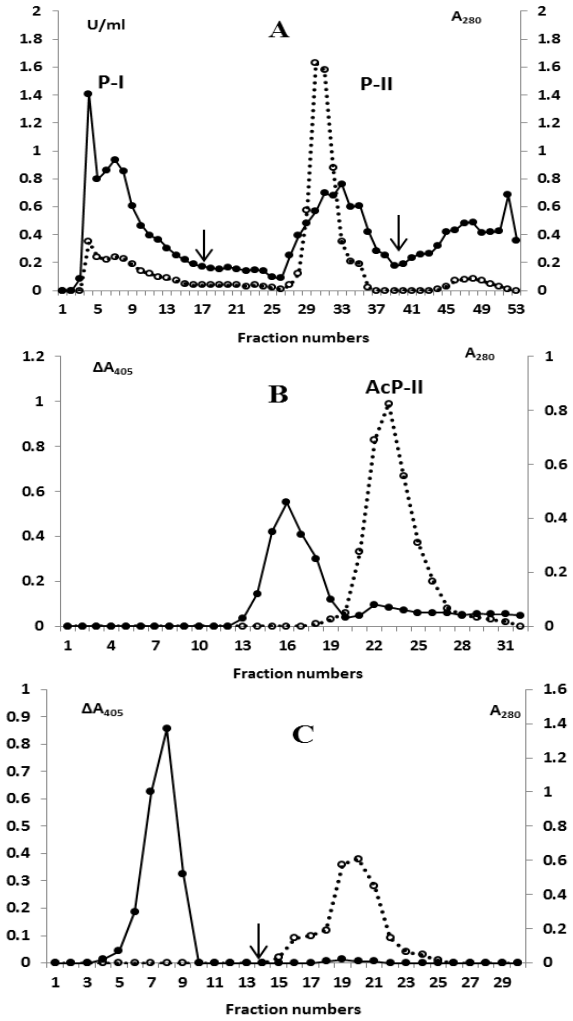

Fig. 1 Purification of acid phosphatase from leaves of germinating *v.radiata* seeds

(A) Ion exchange chromatography on CM-Cellulose.

(B) Gel filtration on Ultrogel AcA 44 column.

(C) Affinity chromatography on Con-A Sepharose 4B column.

Ordinates: Protein at A_{280} nm (●-●); acid phosphatase activity, U/ml or ΔA_{405} (○-○).Arrows show start of various gradients.

The pH dependence of the K_m curve showed (Fig. 6) two inflections, one at pH 5.8 and the other at pH 7.6. These inflections are due to ionization of groups on the enzyme or substrate which may possibly correspond to the pKa_2 of p-nitophenyl phosphate substrate (5.3-5.5) and *pKa* of histidine group on the enzyme (Andrews and Pallavicini, 1973), respectively. Saini and Van Etten, (1978) described that monoanionic form of p-nitophenyl phosphate was hydrolyzed by the enzymes from wheat

germ, human prostate and potato with pKa_2 value of 5.2 found for second ionization constant of p-nitophenyl phosphate and pKa of 7.8 for the ionization of phosphohistidine covalent intermediate. Our conclusion from kinetic data of pH dependence suggests that p-nitophenyl phosphate is hydrolyzed by enzyme involving two groups with pKa^s, of 5.8 and 7.6.

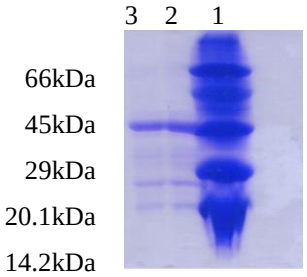

Fig. 2. SDS-polyacrylamide gel electrophoresis of acid phosphatase

Lane1. Standard proteins: Albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa) and lactalbumin (14.2 kDa). Lane 2. 5µl enzyme Lane 3. 5µl enzyme.

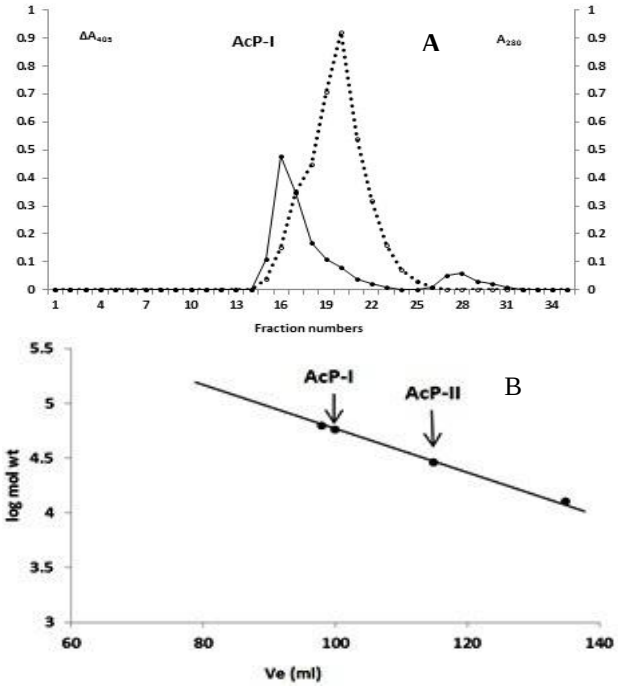

Fig. 3. Estimation of molecular weight of acid phosphatases on Ultrogel AcA 44 column

(A) Elution profile from Ultrogel AcA 44 column of enzyme fraction, P-I obtained from CM-Cellulose chromatography.

(B) Linear graph of log molecular weight versus elution volumes of standard proteins. The standard proteins are albumin (Mr = 66 kDa), V_e 98 ml; carbonic anhydrase (Mr = 29 kDa), V_e 115 ml; cytochrome c (Mr = 12.4 kDa), V_e 135 ml. Arrows indicate the molecular weight of AcP-I and AcP-II isoenzymes, 58 kDa and 29 kDa, respectively.

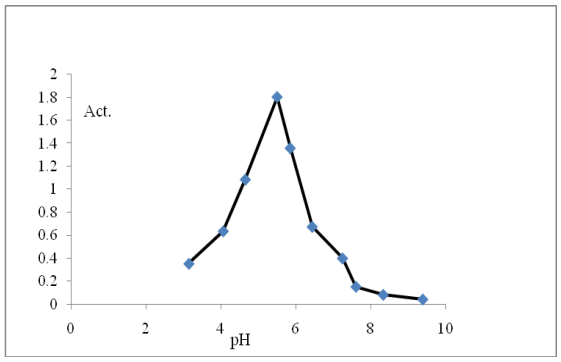

Fig. 4. Optimum pH of acid phosphatase from leaves of *v. radiata*

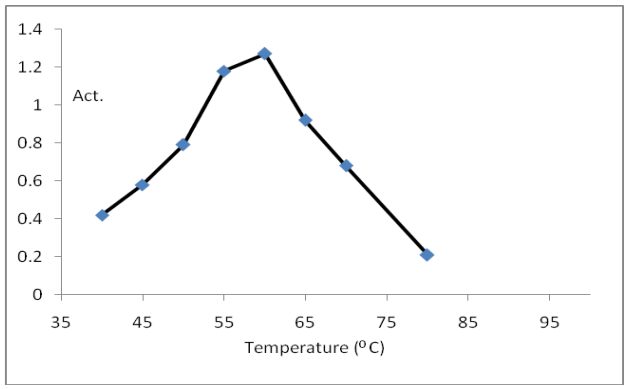

Fig. 5. Optimum temperature of acid phosphatase from leaves of *v. radiata*

The participation of histidine as a part of an active site was supported by enzyme-iodoacetate reaction in time dependent manner. As previously shown in table 5, the reaction of enzyme with 5 mM iodoacetate was slow and had little inhibitory effect (10-20 % at all pH values) during 10 min pre-incubation period but prolonged pre-incubation of enzyme at pH 7.2 in the presence of 100 mM iodoacetate was accompanied by complete inactivation (Table 9) and this was consistent with the presence of an active site histidine residue.

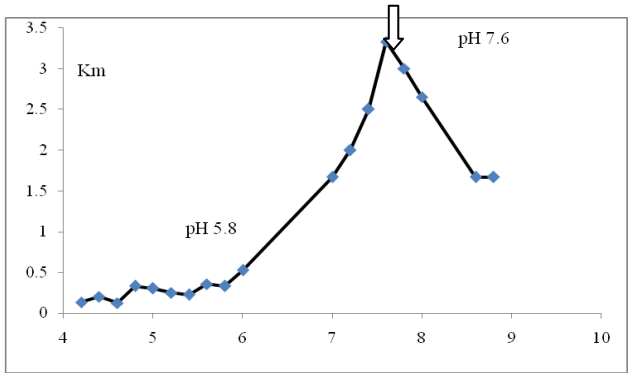

Fig.6. pH dependence of K_m curve for acid phosphatase from leaves of *v. radiata.*

Table 1 Purification of acid phosphatase from 5 g leaves of germinating of *v. radiate* seeds

Steps	Vol. (ml)	T. Act. (U)	T. Prot. (mg)	S.A (U/mg^{-})	P.F.	Rec. %
Extract	15.5	65	224.75	0.29	1	100
Ammonium sulphate (80 %saturation)	16.5	64.35	108.9	0.59	2	99
Dialysis	16.5	63	146.85	0.43	1.48	97
CM-Cellulose Chromatography. (P-II)	38	31.5	45.6	0.69	2.38	48
Concentration by ultrafiltration	5	29.3	40	0.73	2.5	45
Ultrogel AcA 44 Chromatography of P-II. (AcP-II)	35	4.2	2.45	1.714	5.91	6.5
Con-A Sepharose chromatography (AcP-II)	5	2.5	0.1	25	86	3.8

Vol. volume; T.Act. total activity; T.Prot. total protein; S. A. specific activity; P.F. purification factor; Rec. recovery.

Table 2. Substrate specificity of acid phosphatase from the leaves of v. radiata

Substrates	% activity
p-Nitrophenyl phsphate	100
Phenyl phopshate	64.7
α- Naphthyl phosphate	11.3
β- Naphthyl phosphate	63
α-Glycerophosphate	17.8
β- Glycerophosphate	35
Phosphotyrosine	80
Phosphoserine	18
Phosphothreonine	15.4
Glucose-1-phosphate	15
Glucose-6-phosphate	13,6
cAMP	11.5
cGMP	22.4
GMP	27.2
ADP	1.5
ATP	48
FMN	19.7

The enzyme activity was expressed as a percent of that of the same enzyme towards p.nitrophenyl phosphate as 100.

Table 3. Effect of inhibitors on the acid phosphatase activities

Inhibitors	Type of inhibition	*(Ki)* 29 kDa enzyme from leaves of *v.radiata*	*(Ki)* 29 kDa enzyme from seedlings of *v.radiata*
Phosphate	Competitive	5 mM	3.5 mM
Vanadate	Competitive	5 µM	11.5 µM
Fluoride	Non- competitive	0.3 mM	0.6 mM
Molybdate	Mixed type	6-10 µM	3 µM
Zn^{++}	Non- competitive	4 mM	16 mM
Hg^{++}	Non- competitive	13 µ M	30 µ M

Table 4a. Inhibition by metal ions in the presence or absence of EDTA

Metal ions (12.5 mM)	without EDTA treatment Act.	% Act.	with EDTA (20 mM) Act.	% Act.	Recovery (% Act.)
No metal ions (control)	1.264	100	1.302	100	-
Al^{+3}	0.25	19.7	0.962	73.8	54.1
Zn^{+2}	0.273	21.6	1.138	87.4	65.8
Hg^{+2}	0.00	0.00	0.158	12.1	12.1

Table 4b Inhibition by metal ions in the presence or absence of β-merceaptoethanol

Metal ions (12.5 mM)	without β-merceaptoethanol		with β-merceaptoethanol (10 mM)		Recovery (% Act.)
	Act.	% Act.	Act.	% Act.	
No metal ions (control)	0.73	100	0.77	100	-
Al^{+3}	0.12	16.4	0.12	15.6	no
Zn^{+2}	0.17	23.3	0.13	16.9	no
Hg^{+2}	0.00	0.00	0.00	0.00	no

Table 5: Effect of some SH- reacting compounds on the acid phosphatase activity

pH	Iodoacetic acid			Iodoacetamide			N-ethylmaleimide			*p*-hydroxymercuroben-zoate.		
	With 5mM	Without (Control)	% inhibition	With 10mM	Without (Control)	% inhibition	With 10mM	Without (Control)	% inhibition	With 0.5mM	Without (Control)	% inhibition
	Act. (ΔA_{405})	Act. (ΔA_{405})		Act. (ΔA_{405})	Act. (ΔA_{405})		Act. (ΔA_{405})	Act. (ΔA_{405})		Act. (ΔA_{405})	Act. (ΔA_{405})	
3.14	0.92	1.1	16	1.174	1.328	11.6	-	1.407	-	-	-	-
4.06	1.029	1.26	18	1.279	1.508	15	1.144	1.459	21.5	-	-	-
4.65	1.075	1.296	17	1.171	-	-	1.248	1.042	19.8	-	-	-
5.4	1.18	1.416	16.6	1.41	1.64	14	1.383	1.843	25	-	-	-
5.5	1.193	1.48	20	1.446	1.608	10	1.456	1.852	21.4	1.102	1.343	18
5.85	1.31	1.5	13	1.431	1.85	22.6	1.522	1.944	21.7	-	-	-
6.2	1.41	1.731	18	1.52	1.713	11.2	1.52	1.872	14.7	-	-	-
6.44	1.595	1.67	-	1.521	1.637	7	1.573	1.763	10.8	-	-	-
7.25	1.511	1.788	16	1.66	1.758	5.5	1.511	1.85	11.3	-	-	-
8.34	1.49	1.69	12	1.525	1.764	13.5	1.49	1.73	14	-	-	-

Table 6. Effect of different concentrations of SH-protecting or reducing agents on the enzyme activity

Concentration (mM)	DTT		β-mercaptoethanol	
	Activity (ΔA_{405})	Activity (%)	Activity (ΔA_{405})	Activity (%)
0	1.44	100	1.20	100
10	1.492	106.6	1.26	105
20	1.513	107.8	1.226	102
50	1.548	110.5	1.209	102
100	1.539	110.0	1.225	102
200	1.375	95.5	1.08	90
500	1.278	91.3	1.02	85

SH-protecting or reducing agents were added to the enzyme solution at the same time as the substrate

Table 7. Effect of prolonged exposure to 50 mM SH-protecting/reducing agents on the acid phosphatase enzyme

Days	0 (% Activity)	5 (% Activity)	11 (% Activity)	14 (% Activity)	17 (% Activity)
H_2O	100	100	100	100	100
DTT	105	105	103	101	96
β-mercaptoethanol	102	105	101	102	99
Ascorbic acid	95	17	20	14	0

The enzyme was pre-incubated in 0.05 M SH-protecting / reducing agents at 4°C at pH 5.5 for various days. The aliquots were withdrawn and residual activity was determined as usual.

Table 8. pH dependence of the hydrolysis of *p*-nitrophenyl phosphate by acid phosphatase from leaves of germinating *v. radiata*

pH	V_{max} (U/mg)	K_m (mM)	V_{max} / K_m
3.0	3.7	1.11	3.33
3.5	12.5	0.20	62.5
4.0	19.4	0.18	107.77
4.2	22	0.133	165.41
4.4	22	0.200	110.0
4.6	28.6	0.281	101.78
4.8	29.4	0.333	88.29
5.0	30.1	0.303	99.34
5.2	31.14	0.315	98.86
5.4	33.02	0.295	111.93
5.6	35.52	0.357	99.49
5.8	25.36	0.333	76.16
6.0	29.58	0.526	56.36
7.0	35.52	1.670	21.27
7.2	14.8	2.000	7.4
7.4	12.6	2.500	5.04
7.6	8.88	3.330	2.67
7.8	11.1	1.428	7.77
8.0	7.8	2.500	3.12
8.2	8.88	2.500	3.55
8.4	5.0	3.330	1.50
8.6	8.0	1.666	4.80
8.8	6.56	1.666	3.94

(i) V_{max} / K_m (specificity constant) values are almost high in range of pH4 to 5.6 and progressively decreased from above pH 5.6.

Table 9. Inhibition of enzyme with 0.1M iodoacetate at pH 7.2 in function of time

Time	Activity (%)
0 min	100
2min	55
1h	15
2h	9.3
4h	4
24h	1.1
96h	0

Enzyme solution adjusted to pH 7.2 was mixed with equal volume of 0.2M iodoacetate adjusted to pH 7.2 by addition of solid Tris. The mixture was incubated at room temperature in dark and 0.1ml aliquots were withdrawn at various time intervals for assay of enzyme activities. Similar incubation of a control with no added iodoacetate was run.

Conclusions: In this study, low molecular acd phosphatase isoenzyme of (29 kDa) from the leaves of germinating *v. radiata* seeds has been purified and biochemically characterized. The enzyme purification,

electrophoretic pattern, biochemical properties and some other kinetic study reveal that AcP-II from leaves and enzyme isolated from germinating seeds of *v. radiata* are very similar. However, the sequencing data of both 29 kDa enzymes needs to be resolved. No -SH groups are involved in enzyme catalysis but histidine may constitute a part of an active site. Since the enzyme was found insensitive to tartrate inhibition, it may be recognized as a tartrate resistant acid phosphatase class.

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