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RESEARCH ARTICLE

Isolation, purification, characterization and some kinetic properties of acid phosphatase from mungbean [*vigna radiata*] leaves

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Abstract

The present work was carried out on "Isolation, Purification, Characterization and Some Kinetic Properties of Acid Phosphatase from Mungbean [*Vigna radiata*] Leaves". The acid phosphatase (EC. 3.1.3.2) has been noticed and purified from leaves of *vigna radiate* through ammonium sulphate precipitation, DEAE-Cellulose chromatography and concanavalin A-Sepharose 4B chromatography. The specific activity of 1291 nkat.mg-^{1}of protein was obtained with recovery of nearly 1%. About 222 times purification was achieved. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) resolved two bands of acid phosphatase corresponding to molecular weights of 29 kilo Dalton (kDa) and 18 kDa. The molecular weights of native enzyme determined by gel filtration on calibrated Sephadex G-100 column were found to be 29 kDa and 19 kDa. The apparent Km value of 29 kDaiso enzyme with p-nitrophenyl phosphate (pNPP) as substrate was 0.3 mM and Vmax was 1336 nmol.sec^{-1}.mg^{-1} of protein. 5.5 was the optimal pH for this enzyme and 4-7 was the pH stability. It had optimum temperature of 60^{o}C and temperature stability was 0-50^{o}C. Various phosphorylated compounds were hydrolysed non-specifically by this enzyme. However, pNPP, phenyl phosphate, pyrophosphate, ATP, phosphotyrosine and α- naphthyl phosphate seemed to be hydrolysed preferentially. It was competitively inhibited by phosphate, vanadate while fluoride and zinc showed non-competitive inhibitions; the molybdate revealed an inhibition of mixed competitive and Mercury exhibited an inhibition of un-competitive type. It means that all these six elements are responsible for the inhibition of acid phosphatase of leaves of *vigna radiata*. Metal ions such as Cu^{+2}, Hg^{+2}, Zn^{+2} and Al^{+3} showed strong inhibition while other monovalent and divalent ions like Na^{+}, K^{+}, Pb^{+2}, Ba^{+2}, Cd^{+2}, Mn^{+2},Ca^{+2}, Mg^{+2} and Co^{+2} were determined that they had no effect on the enzyme activity. Modifiers, Reducing agents, Detergents and Anions were also established to have no effect on the enzyme activity. At the 4th day of seedlings formation its specific activity was found maximum.

Introduction

Mungbean [*Vigna radiata*] is a well-known legume in Asian countries. It comprises 17- 26% protein and is considered to be a chief source of proteins in developing countries. It is consumed as a viand, boiled and cooked with vegetables and meat, as well as a dessert, cooked in syrup, or incorporated in bread or cake. It is also widely used to make sprouts for egg rolls and other vegetable dishes. In biological systems, enzymes act in ordered sequences catalyzing hundreds of complex reactions in metabolic pathways. A few of these enzymes are regulatory in nature. A broad study of enzymes involves a study of its molecular structure, physical properties, catalytic properties, thermodynamics, active site and biological properties. For these studies the enzyme has to be purified and studied in vitro. The enzymes are the proteins having catalytic function in biological processes.

Acid phosphatases (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2) are extensively distributed in plants and animals. Many authors have isolated and characterized acid phosphatases from tubers (Gellatly*et al*., 1994; Kusudo *et al*., 2003), seeds (Ullah and Gibson, 1988; Olczak*et al*., 1997; Granjeiro*et al*., 1999), roots (Panara et al., 1990), leaves (Staswick*et al*., 1994) and seedlings (Yenigun and Guvenilir, 2003).

Acid phosphatases are ubiquitous enzymes that show activity against a variety of substrates in vitro, although little is known about their intracellular function. Acid phosphatases (EC. 3.1.3.2) are a group of enzymes that catalyze the hydrolysis of many organic phosphate monoesters and show pH optima on acid side pH 5-6 (Vincent *et al*., 1992). These enzymes are ubiquitous in nature and widely distributed in plants. Plant acid phosphatases are present in various plant tissues and cell organelles. These often occur in multiple forms and can vary in molecular weight, substrate specificity, sensitivity to inhibitors and some kinetic properties (Penheiter *et al*., 1997, Turner and Plaxton, 2001., Zhou *et al*., 2003).Acid phosphatases (EC 3.1.3.2), belong to hydrolases class, have catalytic action in the hydrolysis of various phosphomonesters in acidic medium (pH 5-6) to liberate an inorganic phosphate (Vincent *et al.*, 1992). These are found in animals, plants (Demir*et al*., 2004; Gonnety*et al.*, 2006; Kaida *et al.*, 2008; Tabaldi *et al.*, 2008) and lower organisms like protozoa and fungi. For purification and characterization of acid phosphatase enzymes in leaves a few attempts were made. Acid phosphatase isoenzymes isolated from garlic seedlings (Yenigun and Guvenilir, 2003), common bean seedlings (Tejera-Garcia *et al.*, 2004) and peanut seedlings (Gonnety*et al.*, 2006) were also reported. In recent times the study on the presence of multiple acid phosphatase activity in seedlings of cucumber, radish and rocket salad (Tabaldi*et al*., 2008) was undertaken. The present work deals with the purification of acid phosphatase isoenzyme from the leaves of *Vigna radiata* and its characterization with respect to kinetic parameters, optimum temperature, thermal stability, pH dependence, substrate specificity, molecular weight determination and the effect of metal ions and other substances on its activity.

MATERIALS AND METHODS

Plant material

Seeds of *Vigna radiate* were surface sterilized with 1% (v/v) solution of sodium hypochloride for 10 min, washed three times with distilled water and soaked in water for 3-4 h. After hydration, germination of seeds was performed on moist sand trays during at least 8 days in a room at ambient temperature of 28-35°C. The germinating seeds were daily watered until small plants with leaves were produced.

Chemicals

The important chemicals which were used in this research are Sephadex G-100, p-nitrophenyl phosphate, α-naphthyl phosphate, β-glycerol phosphate, β-naphthyl phosphate, Flavin mononucleotide phosphate, Phenyl phosphate, Bovine serum albumin, Concanavalin A-Sepharose 4B, SDS molecular weight protein markers were purchased from Merck, 0.1M Acetate buffer of pH 5.5, 0.1N KOH solution, Sigma Chemical Co. &Fluka Chemical Co., DEAE Cellulose was obtained from Whatman Biosystem. The chemicals for Polyacrylamide gel-electrophoresis were supplied by Acros Chemical Co. while all other chemicals were of highest purity analytical grade.

Extraction of the enzyme

After germination during the period of 8 days seedlings (whole plants) were collected dailyand refrigerated. After broken by hand the leaves were finely ground with the help of a mortar and pestle and added of 0.1M acetate buffer having pH 5.5 containing 0.1Mm PMSF (phenylmethylsulphonyl fluoride) at the rate of 1g leaves/5ml buffer. The homogenate was centrifuged at 45,000 rpm for 5-10 min. The supernatant collected, was used for enzyme assay and protein concentration. We used grinding of plant material with porcelin mortar and pestle in the extraction buffer for our study and the efficiency of extraction was assessed by determining the total protein content and extraction protocol was finalized.

Protein Determination

Protein concentration was determined by the Lowry method.

Procedure

To precipitate the protein100µl of protein sample was diluted by adding 900µl of distilled water followed by the addition of 170µl of 50% trichloroacetic acid (TCA). Then the mixture was centrifuged at 10,000rpm for 4-5 minutes. The supernatant was removed while the precipitated protein was collected. The precipitate was dissolved in 1.25ml of biuret reagent (composed of 0.9% sodium- potassium tartarate, 0.3% CuSO4. $5H_2O$ and 0.5% KI in 0.2 N NaOH) and mixed by vortex until gets dissolved. Again diluted with 1.25ml distill water and incubated for 10 min for development of a violet color. Absorption was recorded at 546nm on a spectrophotometer. The standard curve was made using bovine serum albumin (Fig.1).In column effluents; the relative protein concentration was estimated by the absorbance at 280nm.

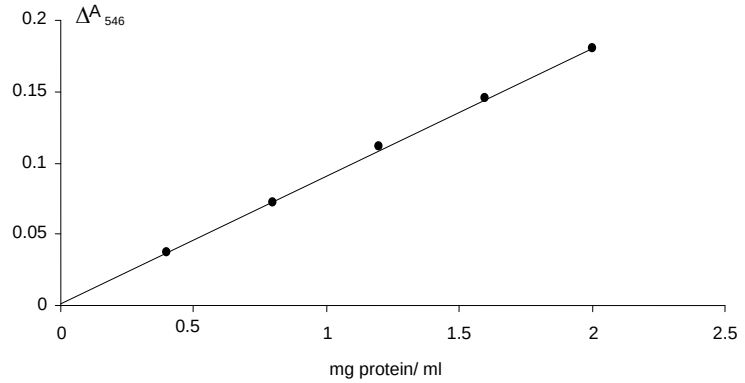


Fig. 1 Standard curve for the determination of protein concentration by Biuret method.

Preparation of gel mixture

Composition of the gel mixture for resolving gel andstacking gel are given below:

(a) Solution for preparation of resolving gel (12%), 0.37 M Tris-HCl pH 8.8

Distilled water	3.35 ml
1.5M Tris-HCl, pH 8.8	2.5 ml
Acrylamide/Bis (30% Stock)	4.0 ml
10% (w/v) SDS Stock	100µl
10% ammonium persulphate	50 µl
TEMED	5 µl
Total volume =	10 ml

(b) Solution for preparation of stacking gel (4%), 0.125M Tris-Hcl pH 6.8

Distilled water	6.1 ml
0.5M Tris-HCl, pH 6.8	2.5 ml
Acrylamide/Bis (30% Stock)	1.3 ml
Degas under vaccum for 3-5 minutes and add	
10% (w/v) SDS Stock	100µl
10% ammonium persulphate50 µl	
TEMED	5 µl
Total volume =	10 ml

All the components except 10% ammonium persulphate and TEMED were mixed in the required amounts for resolving and separating gels separately. Required amount of 10% ammonium persulphate and TEMED were added and thoroughly mixed to the gel mixture before pouring the assembled gel sandwitch.

Acid phosphatase purification from *Vignaradiata* Leaves Extraction

All operations were carried out at the temperature of4°C. Using procedure of Pasqualini *et al.*,1992 and 1997 for enzyme purification. The seeds were grown as described above. Seedlings (whole plants) were collected after germination of 3-4 days and their leaves were homogenized in 0.1M acetate buffer (pH 5.5) at the rate of 1g leaves/5 mL buffer, with mortar and pestle. This was followed by stirring for one hour. The homogenate was centrifuged at 45,000rpm for 1h. The supernatant was collected while pellet was discarded.

RESULTS

Acid phosphatase activity from leaves of *Vigna radiata*

At ambient temperature, *Vigna radiata* seeds were germinated in aired room. When the seedlings (whole plants) were formed, the activity of leaves was checked. On the 4th day of Seedlings formation, specific acid phosphatase activity of leaves was Maximum (6 nkat.mg^{-1}of protein) (Table1 and Fig.2) and gradually decreased after 4th day. On the 7th day, the activity was about half of maximum specific activity (3.2 nkat.mg^{-1} of protein), which indicates that increase in activity of acid phosphatase is dependent on leaves formation. Similar results have been reported by Gonnety*et al*., 2006 in peanut seedlings and Senna*et al*., 2006 in maize seeds.

Table-1: Expression of acid phosphatase activities from V. radiata Leaves.

Days	Activity (nkat / ml)	Protein concentration (mg / ml)	Specific activity (nkat.mg^{-1})
0	11.522	5.7	2.021
1	12.232	5.9	2.073
2	18.362	4.6	3.991
3	21.417	4.0	5.354
4	23.110	3.8	6.081
5	13.856	3.6	3.848
6	10.263	3.7	2.773
7	11.542	3.6	3.206
8	9.178	3.1	2.960

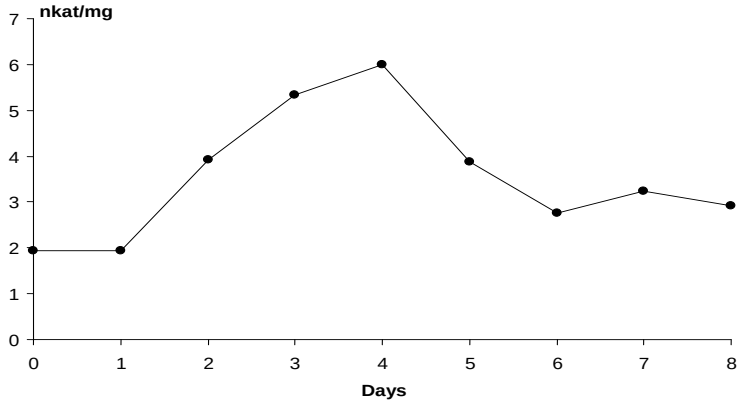


Fig. 2 Expression of Acid phosphatase activities from *V. radiata* leaves.

Purification of an Acid Phosphatase

After flowering leaves were harvested from plants grown in a growth chamber and depodded two times on weekly basis for 5 weeks. Leaves were stored frozen at -80^{0}C. Frozen tissue wrapped in foil was first broken by hand, then

20g was homogenized with a Polytron at 4^0C in 150mL of 50mM Tris-HCl pH 7.6, 1mM EDTA, 30mM ascorbic acid, 1mM DTT, and 3%(w/v) insoluble polyvinylpolypyrrolidone. After filtration through four layers of cheesecloth, the extract was centrifuged at 17,000g for 10 min. The supematant was brought to 2M ammonium sulfate and stirred on ice for 1hour. The supernatant was obtained after centrifugation as before and brought to 2.8Mwith ammonium sulfate. After 1hour on ice the resulting precipitate was collected by centrifugation as described above. The pellet was resuspended in 5ml of 25m Mes pH 6.5, insoluble material was removed by centrifugation(5min at 17,000g), and the supernatant was desalted by gel filtration chromatography on Sephadex G-25 (1.5 X27 cm) in the same buffer. Precipitate which was formed during desalting was seperated by centrifugation. The supematant was loaded on a DEAE Sephadex A-50 column (2.5 x10 cm) equilibrated with 25mM Mes pH 6.5. Usually the first 20ml contained the majority of acid phosphatase activity, which did not bind to the column. This fraction was centrifuged as before and then loaded directly on a hydroxylapatite column (1 X 18 cm) equilibrated with 10mM Na phosphate pH 7.3. The column was eluted with a 200ml linear gradient of Na phosphate (from 10-400m pH 6.7). The single activity peak was dialyzed against 4L of 10mM $NaP0_4$ (pH 7) and loaded onto a CM Sephadex C-50 column (0.8 x 30 cm), which was developed with a linear gradient of NaCl to 0.2M**.** NaN_3 (0.2%, w/v) was included in all chromatography buffers as an antimicrobial agent. All steps beginning with tissue homogenization were done at 4^0C, with the exception of hydroxylapatite chromatography, which was conducted at room temperature to avoid precipitation of Na phosphate.

Acid phosphatase was purified 222-fold from the leaves of 3-4 days old *V. radiata*. The purification steps are presented in table2 and their elution profiles of chromatography are shown in Fig.3 (a and b). The enzyme was obtained with specific activity of 1291nkat.$mg^{-1}$of protein and recovery of 0.7%. A great decrease in the total activity occurs during ammonium sulphate (AS) precipitation at 70% saturation level and DEAE-Cellulose chromatography steps resulting in a very low recovery of enzyme. The enzyme might be glycoprotein as this was bound to Con-A column and was eluted by α-D-methylmannoside.

The homogeneity of the enzyme was checked on SDS-polyacrylamide gel eletrophoresis (12%). Two bands were detected and their molecular weights were estimated to 29kDa and 18kDa (Fig.4a, b). The gel filtration experiment of crude extract saltedout from ammonium sulphate (30-60% saturation) showed one peak of acid phosphatase (V_e, 126 ml) but slight shoulder on the descending part of the peak (V_e, 135 ml) was observed. The elution profile is shown in Fig.5a. Apparent molecular weight of these two enzymes obtained by gel filtration on calibrated Sephadex G-100 column was estimated to be 29kDa and 19kDa (Fig.5b) which supports that acid phosphatase from *V. radiata* has two isoenzymes with molecular weights of 29kDa and 18kDa. Two isoenzymes were separated from each other by reverse-phase HPLC. Two peaks A and B are shown in Fig.6. The peak A contained homogeneous acid phosphatase. When homogeneity of this isoenzyme was checked on SDS-PAGE, single band corresponding to 29kDa was observed (Fig.7). The expected band of peak B corresponding to 18kDa was not detected on SDS-PAGE.

Table-2: Various steps involved in purification of acid phosphatase from 130g leaves of *Vignaradiata*

Steps	Volume. (ml)	TotalActivity. (nkat**)**	TotalProtein**.** (mg)	SpecificActivity. (nkat.mg^1)	Purificat.Factor.	Recovery. (%)
Extract	925	20700	3561	5.81	1	100
AS(30%saturation)	935	19050	3500	5.44	0.94	92
AS(70%saturation)	35	7310	306	23.89	4.11	35.3
DEAE–Cellulose Chromatography(after pool,concentration & dialysis)	12	473	6.5	72.77	12.52	2.3
ConA-Sepharose4B Chromatography	4	155	0.12	1291.67	222.32	0.75

Protein concentration was determined by Lowry method.

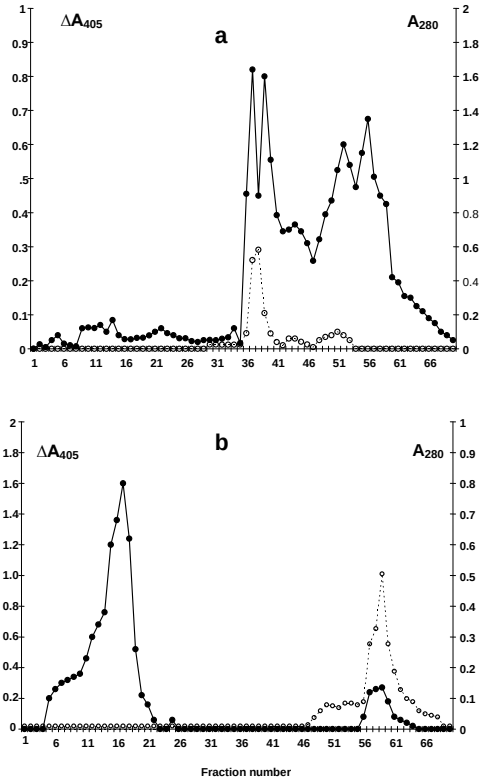


Fig. 3 (a) Ion-exchange chromatography on DEAE-Cellulose.
(b) Affinity chromatography on Con-A Sepharose 4B column.
Ordinates: Protein at 280nm ((●—●);
acid phosphatase activity, ΔA_{405}(o····o).

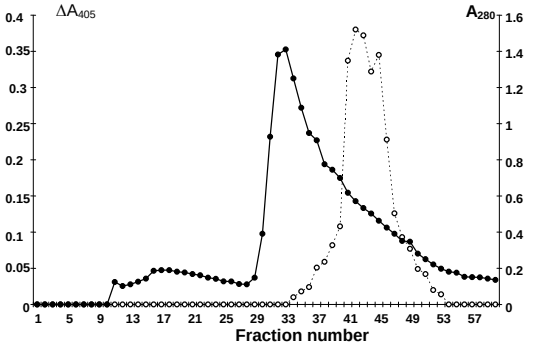


Fig.5 (a) Eluton profile from Sephadex G-100.
Ordinates: Protein at 280nm ((●—●);
acid phosphatase activity ΔA_{405}(o····o).

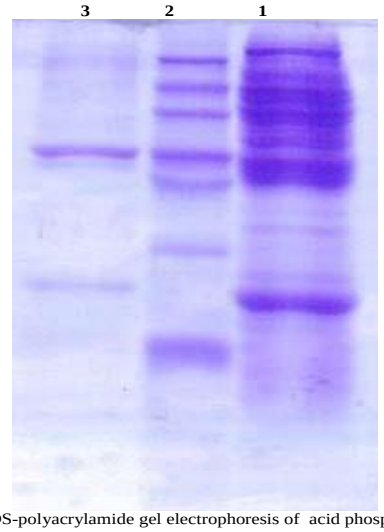


Fig. 4(a) SDS-polyacrylamide gel electrophoresis of acid phosphatase enzyme.
Lane 1 5µl non-bound protein peak sample from Con A column.
Lane 2 Protein markers used from top to bottom are :
(1) Albumin (66 kDa), (2) ovalbumin (45 kDa),
(3) glycerolaldehyde-3-phosphate dehydrogenase (36 kDa),
(4) carbonic anhydrase (29 kDa), (5) trypsinogen (24 kDa),
(6) trypsin inhibitor (20.1 KDa) and (7) lactalbumin (14.2 kDa).
Lane 3 5µl of acid phosphatase isoenzymes peak from mannoside elution.

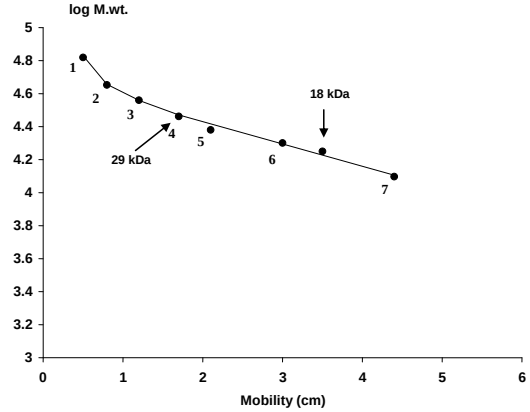


Fig. 4 (b) Linear graph of log molecular weight versus mobility of stained bands of proteins markers 1 to 7.
Arrows indicate the molecular weight of isoenzymes.

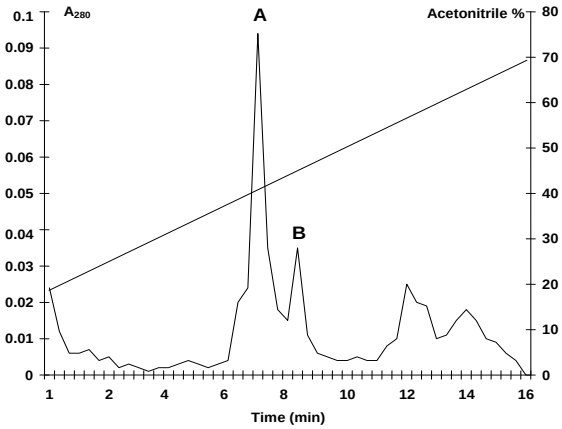


Fig. 6 Separation of acid phosphatase isoenzymes in reverse-phase chromatography.
Flow rate, 1ml/min; Back pressure, 420 psi; Absorbance at 280 nm (—); Elution gradient (—)· Peaks A and B are two isoenzymes.

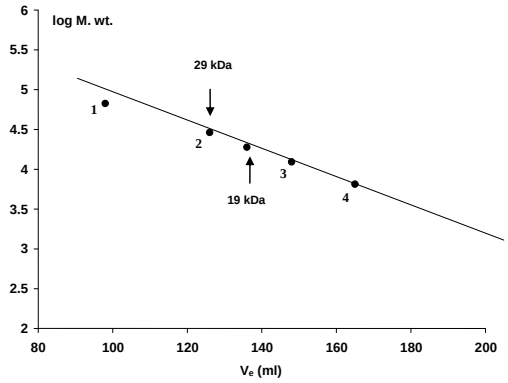


Fig. 5 (b) Linear graph of log moleculr weight versus eluton volumes of standard proteins (1) Bovine serum albumin (M_r 66,000), V_e 98ml; (2) Carbonic anhydrase (M_r 29,000), V_e 126ml; (3) Cytochrome c (M_r 12,400), V_e 148ml; (4) Aprotinin (M_r 6,500).
Arrows show the molecular weight of isoenzymes.

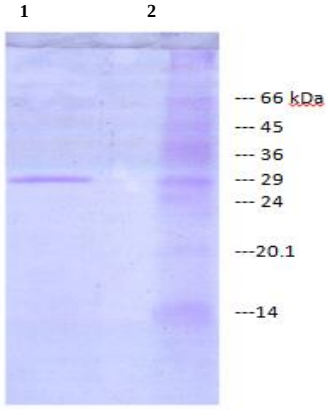


Fig. 7 SDS-polyacrylamide gel electrophoresis of Peak A obtained from HPLC.
Lane 1 Protein markers as mentioned in Fig 4a.
Lane 2 10µl of isoenzyme (Peak A).

Kinetic properties

Acid phosphatase from leaves had optimum pH at 5.5 (Fig.8) which was consistent with other pH optima, 5.0-6.0 (Jagiello*et al.*, 1992., Ferreira *et al.*, 1998). The pH stability was found between pH 4 and 7 (Fig.9). The enzyme had optimum temperature of 60°C (Fig.10) and showed temperature stability around 50°C (Fig.11).The enzyme was stable at 40- 50°C.At 60°C the enzyme lost 5% activity after 5 min, at 70°C the enzyme lost 40% activity after 5 min and at 80°C the enzyme lost was 98% after 5 min (Fig.12). This attitude seems to be a general character of plant acid phosphatases. To further evaluate the kinetic properties of acid phosphatase, a time course experiment was conducted under the conditions of enzyme assay. The product formation was linear with time (Fig.13) and velocity was directly proportinol to enzyme concentration (Fig.14). The $K_{m\ obtained}$ was 0.3mM and V_{max} was 1336nmol.sec^{-1}.mg^{-1} of protein.

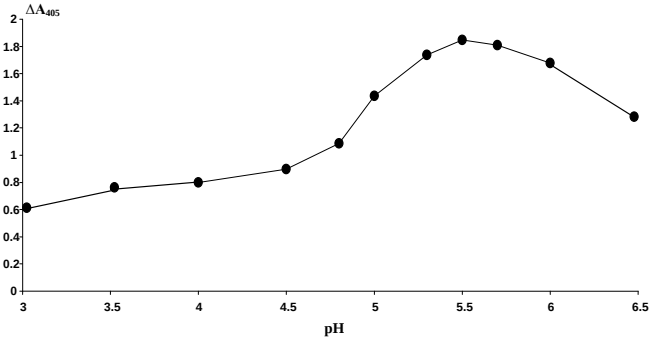


Fig. 8 pH optimum of acid phosphatase activity.

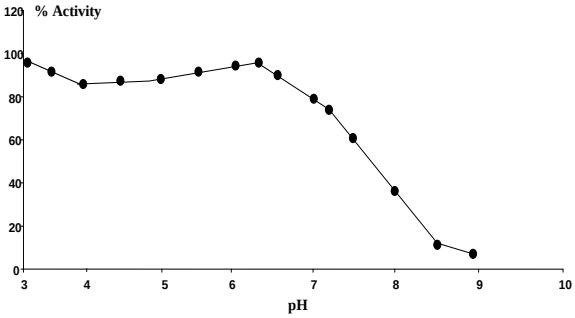


Fig. 9 pH stability of acid phosphatase.

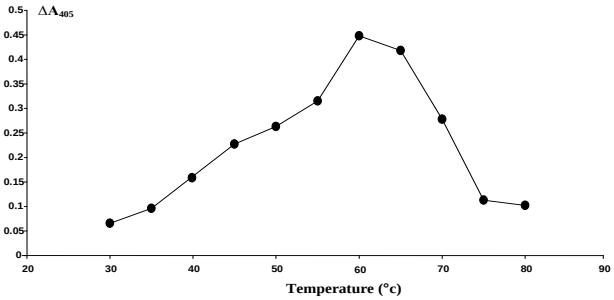


Fig. 10 Optimum temperature of acid phosphatase.

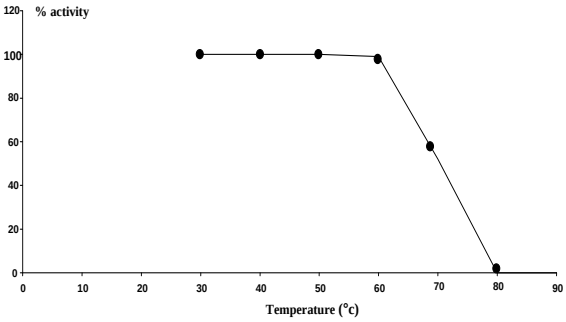


Fig. 11 Temperature stability of acid phosphatase

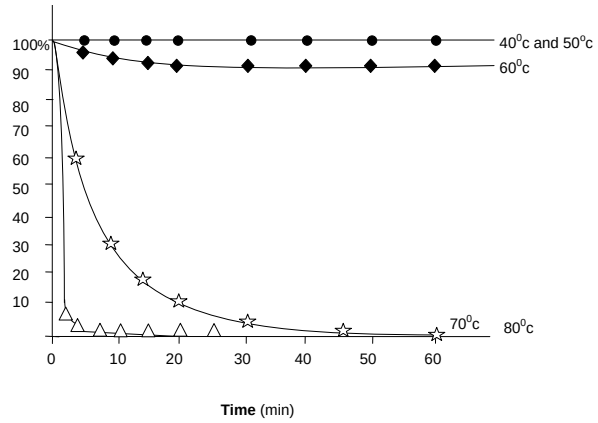


Fig. 12 Thermal stability of acid phosphatase at various temperatures.

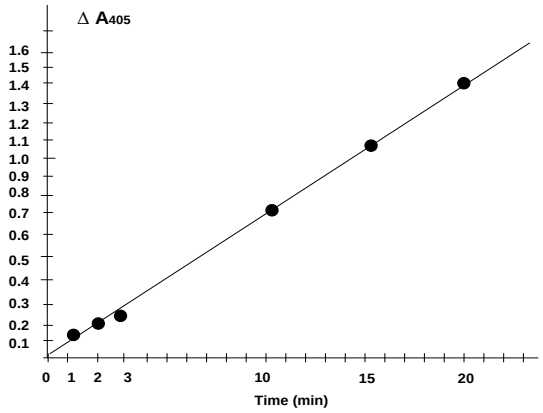


Fig. 13 Acid phosphatase activity in function of time

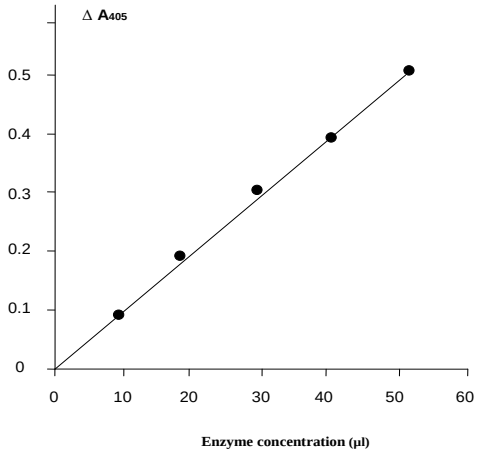


Fig. 14 Acid phosphatase activity in function of [E]

Table-3:Substrate specificity of *V. radiata* acid phosphatase

Substrates	% activity
pNPP	100
Phenyl phosphate	92
α-glycerophosphate	41
β-glycerophosphate	42
α-naphthyl phosphate	95
β-naphthyl phosphate	21
FMN	0
Ribulose-5-phosphate	0
Glucose-1-phosphate	22
Glucose-6-phosphate	48
Fructose-1-phosphate	18
Phosphothreonine	31
Phosphoserine	18
Phosphotyrosine	71
AMP	41
ATP	73
Sodium pyrophosphate	101

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

Table-4:Effect of metal ions (cations) on acid phosphatase of leaves of *Vigna radiata*

Metal ions	Concentration	Acid phosphatase of leaves (% Activity)
H_2O	0 mM	100
NaCl	5 mM	101
KCl	5 mM	101
$Pb(CH_3COO)_2$	5 mM	82
$CoCl_2$	5 mM	96
$BaCl_2$	5 mM	100
$CdCl_2$	5 mM	99
$MnCl_2$	5 mM	82
$CaCl_2$	5 mM	102
$MgCl_2$	5 mM	101
$ZnCl_2$	5 mM	2
Cu_2SO_4	5 mM	20
$AlCl_3$	5 mM	13
$HgCl_2$	5 mM	3

The results were expressed as relative percentage of activity respect to the control reaction without ion added.

Table-5: Effect of modifiers on acid phosphatase of leaves of *V. radiata*

Modifiers	Acid phosphatase of leaves (% Activity)
H_2O	100
Ethanol	107
Glycerol	103
Methanol	112
Acetone	118
PMSF	63
EDTA	99
Ascorbic Acid	92

Table-6: Effect of reducing agents on acid phosphatase of leaves of *V. radiata*

Reducing agents	Acid phosphatase of leaves (% Activity)
H_2O	100
β -mercaptoethanol	101
p-Hydroxymercuribenzoate	102
Iodoacetic acid	102
Glutathion	102
DTT(Dithiothreitol)	101

Table-7: Effect of detergents on acid phosphatase of leaves of *V. radiata*

Detergents	Acid phosphatase of leaves (% Activity)
H_2O	100
Tween-20	96
Triton X-100	101

Table-8: Effect of anions on acid phosphatase of leaves of *V. radiata*

Anions	Concentration	Acid phosphatase of leaves (% Activity)
H_2O	0 mM	100
$NaHCO_3$	5 mM	111
$NaNO_3$	5 mM	108
Na_2SO_4	5 mM	108
Na_2CO_3	5 mM	119

Table-9: Effect of phosphate inhibitors on acid phosphatase of leaves of *V. radiata*

Phosphate inhibitors	Concentration	Acid phosphatase of leaves (% Activity)
H_2O	0 mM	100
Na_3Vo_3	5 mM	11
Na_3Po_4 (High conc.)	5 mM	26
Na,K.Tartrate	5 mM	105
NaF	5 mM	10
Na_2MoO_4	5 mM	6
Na.Citrate.$2H_2o$	5 mM	109
Na_3Po_4 (Low conc.)	5 mM	107

Table-10:Effect of phosphate inhibitor with function PO_4 Conc.(100mM) on acid phosphatase ofleaves of *V. radiate*

KH_2PO_4 conc.	Concentration	Acid phosphatase of leaves (% Activity)
0µl(H_2O)	0 mM	100
10µl	1 mM	104
20µl	2 mM	107
30µl	3 mM	107
40µl	4 mM	108
50µl	5 mM	110
100µl	10 mM	111

Table-11: Effect of phosphate inhibitor with function PO_4 Conc. (1000mM) on acid phosphatase of leaves of *V. radiata*

KH_2PO_4 conc.	Concentration	Acid phosphatase of leaves (% Activity)
0µl(H_2O)	0 mM	100
1µl	1 mM	107
2µl	2 mM	107
3µl	3 mM	107
4µl	4 mM	106
5µl	5 mM	97
10µl	10 mM	81
20µl	20 mM	56
30µl	30 mM	43
40µl	40 mM	34
50µl	50 mM	26
100µl	100 mM	19
200µl	200 mM	9

Table-12: Effect of fluoride inhibition with different Conc. on acid phosphatase of leaves of*V. radiate*

NaF Conc.	Concentration	Acid phosphatase of leaves (% Activity)
0µl(H_2O)	0 mM	100
10µl	1 mM	26
20µl	2 mM	17
30µl	3 mM	13
40µl	4 mM	13
50µl	5 mM	11
60µl	6 mM	11
70µl	7 mM	10
80µl	8 mM	10
90µl	9 mM	9
100µl	10 mM	9

Table-13: Effect of molybdate inhibition with different Conc. on acid phosphatase of leaves Of *V. radiata*

Na_2MoO_4 Conc.	Concentration	Acid phosphatase of leaves (% Activity)
0µl(H_2O)	0 mM	100
10µl	0.1 mM	9
20µl	0.2 mM	8
30µl	0.3 mM	7
40µl	0.4 mM	7
50µl	0.5 mM	7
60µl	0.6 mM	7
70µl	0.7 mM	7
80µl	0.8 mM	7
90µl	0.9 mM	7
100µl	1.0 mM	7

Table-14: Effect of vanadate inhibition with different Conc. on acid phosphatase of leaves of*V. radiata*

Na_3VO_3 Conc.	Concentration	Acid phosphatase of leaves (% Activity)
0µl(H_2O)	0 mM	100
10µl	0.1 mM	35
20µl	0.2 mM	21
30µl	0.3 mM	16
40µl	0.4 mM	14
50µl	0.5 mM	13
60µl	0.6 mM	12
70µl	0.7 mM	11
80µl	0.8 mM	11
90µl	0.9 mM	10
100µl	1.0 mM	9

Table-15: Effect of zinc inhibition with different Conc. on acid phosphatase of leaves Of *V. radiate*

$Zncl_2$ Conc.	Concentration	Acid phosphatase of leaves (% Activity)
0µl(H_2O)	0 mM	100
10µl	1 mM	65
20µl	2 mM	47
30µl	3 mM	35
40µl	4 mM	29
50µl	5 mM	25

Table-16: Effect of mercury inhibition with different Conc. on acid phosphatase of leaves Of *V. radiate*

$Hgcl_2$ Conc.	Concentration	Acid phosphatase of leaves (% Activity)
0µl(H_2O)	0 mM	100
10µl	0.1 mM	57
20µl	0.2 mM	44
30µl	0.3 mM	33
40µl	0.4 mM	28
50µl	0.5 mM	24
60µl	0.6 mM	21
70µl	0.7 mM	20
80µl	0.8 mM	17
90µl	0.9 mM	16
100µl	1.0 mM	14

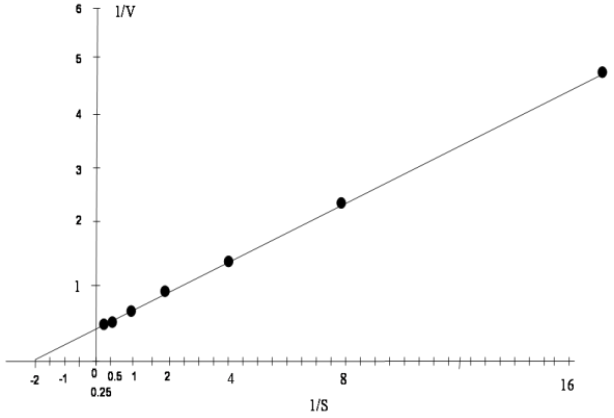
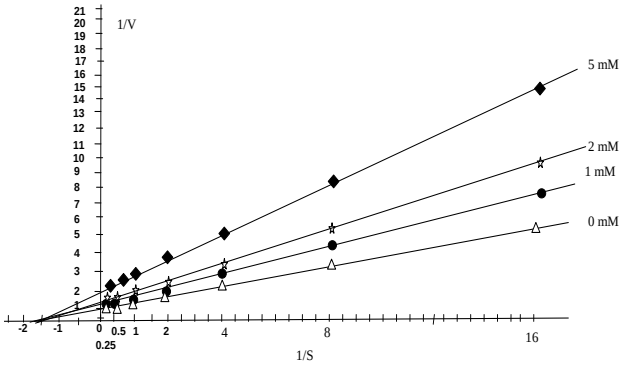


Fig. 15 Km & Vmax determination of Acid Phosphatase of *Vigna radiata* Leaves.



17 Non-Competitive Inhibition of acid phosphatase from *V. radiata* leaves. Lineweaver-Burk plot of 1/ V versus 1/ S. In the absence of 0mM ( ), presence of 1mM ( ), 2mM ( ) and 5mM ( ), zinc ($ZnCl_2$) inhibitor.

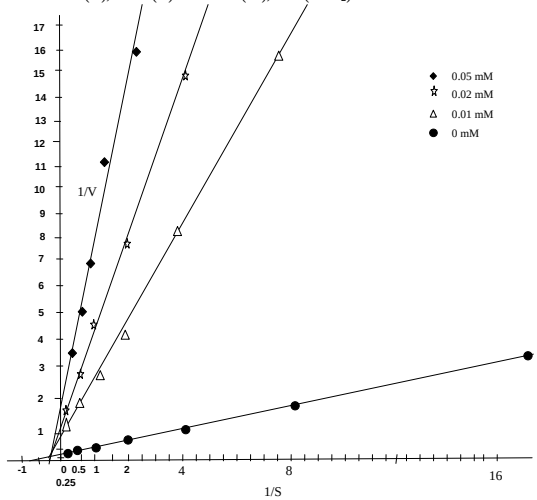


Fig. 20 Mix-Competitive Inhibition of acid phosphatase from *V. radiata* leaves. Lineweaver-Burk plot of 1/ V versus 1/ S. In the absence of 0mM ( ), presence of 0.01mM ( ), 0.02mM ( ) and 0.05mM ( ), molybdate (Na_2MoO_4) inhibitor.

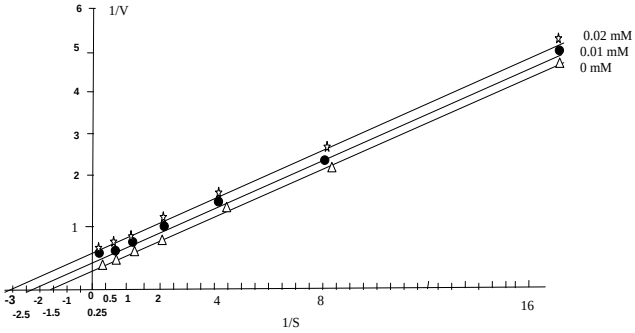


Fig. 16 Un-Competitive Inhibition of acid phosphatase from *V. radiata* leaves. Lineweaver-Burk plot of 1/ V versus 1/ S. In the absence 0f 0mM ( ), presence 0f 0.01mM ( ) and 0.02mM ( ), mercury ($HgCl_2$) inhibitor.

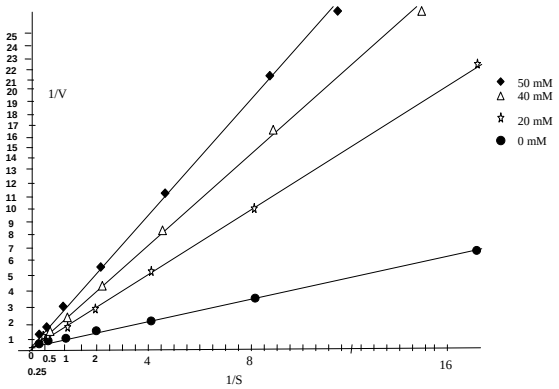


Fig. 18 Competitive Inhibition of acid phosphatase from *V. radiata* leaves. Lineweaver-Burk plot of 1/ V versus 1/ S. In the absence of 0mM ( ), presence of 20mM ( ), 40mM ( ) and 50mM ( ), phosphate (KH_2PO_4) inhibitor.

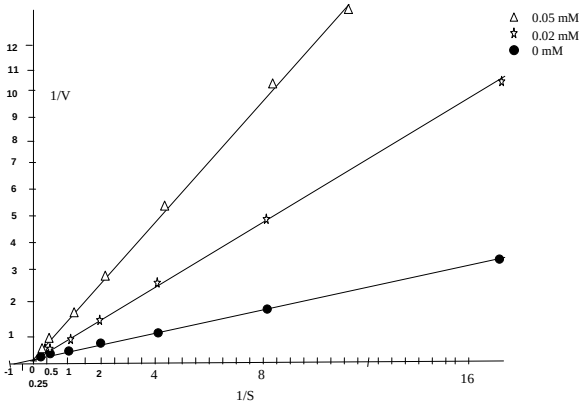


Fig. 19 Competitive Inhibition of acid phosphatase from *V. radiata* leaves. Lineweaver-Burk plot of 1/ V versus 1/ S. In the absence of 0mM ( ), presence of 0.02mM ( ) and 0.05mM ( ), vanadate (Na_3VO_3) inhibitor.

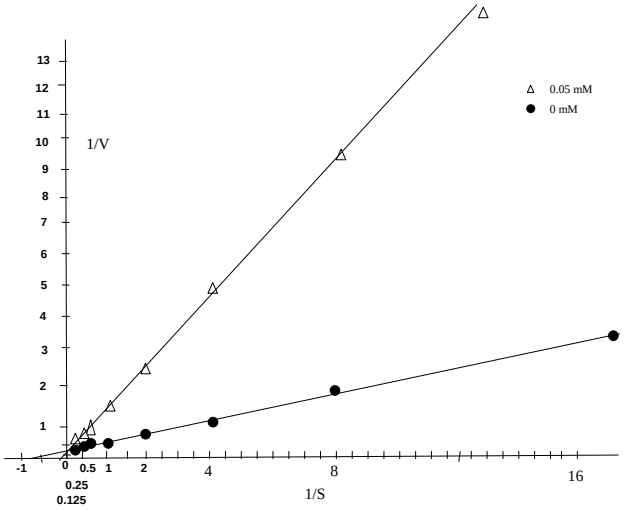


Fig. 21 Competitive Inhibition of acid phosphatase from *V. radiata* leaves. Lineweaver-Burk plot of 1/ V versus 1/ S. In the absence of 0mM (●), presence of 0.05mM (Δ), vanadate (Na_3Vo_3) inhibitor.

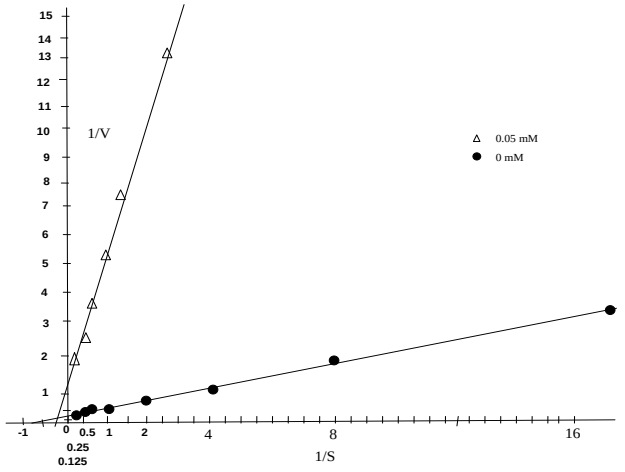


Fig. 22 Mix-Competitive Inhibition of acid phosphatase from *V. radiata* leaves. Lineweaver-Burk plot of 1/ V versus 1/ S. In the absence of 0mM (●), presence of 0.05mM (Δ), molybdate (Na_2MoO4) inhibitor.

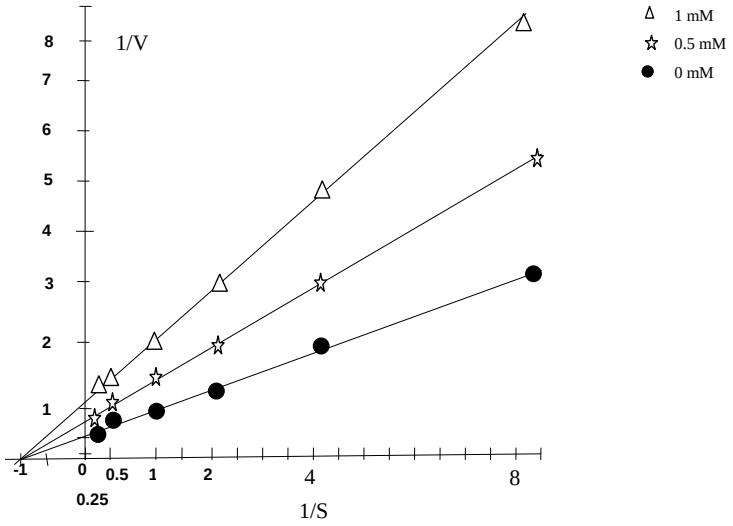


Fig. 23 Non-Competitive Inhibition of acid phosphatase from *V. radiata* leaves. Lineweaver-Burk plot of 1/ V versus 1/ S. In the absence of 0mM (●), presence of 0.5mM (☆) and 1mM (Δ), fluoride (NaF) inhibitor.

DISCUSSION

During the seedlings (whole plants) formation, synthesis or activation of enzymes takes place which normally break down macromolecular organic reserves in seedlings into simpler molecules. The acid phosphatase is one of the enzymes which play ankey role in the seedlings formation. Acid phosphatases are constitutively expressed in leaves during seedlings formation and hence their activities increased with the first stage of leaves formation to release phosphates for the growing embryo (Biswas and Cundiff, 1991). In the present study, the specific acid phosphatase activity increased and reached the upper limit on the fourth day of *V. radiate* leaves formation at temperature between 28-35°C. Gonnety*et al*., (2006) noticed that maximum specific acid phosphatase activity of peanut seedlings was recorded on the 5th day for germination temperature below 25°C. Similarly, Prazeres *et al.*, (2004) showed that the maximum activity of soybean seedlings was appeared on the 6th day of germination at temperature of 28°C. Hegeman and Grabau (Hegeman and Grabau, 2001) also determined that 3-9 days were the mean time for germination of peanut seedlings regarding acid phosphatase activity. In our case, seedlings of 2-5 days of age could be the reasonable time considered for swift growth in relation to acid phosphatase activity (Table 1) but we used 3-4

days of seedlings regarding their size for the purification. The enzyme was purified by ammonium sulphate fractionation, ion-exchange chromatography and affinity chromatography from *V. radiata* seedlings. 222-folds purification was resulted with specific activity of 1291 nkat.mg^{-1} of protein. SDS- PAGE exhibited two bands of protein with molecular weights of 29 kDa and 18 kDa and by gel filtration, the native enzyme had 29 kDa and 19 kDa, indicating the existence of multiple forms of acid phosphatase in *V. radiata* seedlings as reported for multiple forms of acid phosphatases from many other sources (Tamura *et al*., 1982 ., Ullah and Gibsson, 1988., Panara*et al*., 1990., Biswas and Candiff, 1991., Kaneko *et al*., 1990., Pasqualini *et al*, 1997., Tso and Chen, 1997). Reverse phase HPLC was used in the last stage of purification. Since acetonitrile caused denaturation of the enzyme, therefore HPLC step was not included in this scheme. However, the major protein peak (A) obtained from HPLC was found to be homogeneous on SDS- PAGE and moved as a single protein band of molecular weight of 29 kDa while minor protein peak (B) corresponding to 18 kDa was not visualized as a protein band on SDS- PAGE confirming the existence of two isoenzymes with molecular weight of 29 kDa and 18 kDa in *V. radiata* leaves. Molecular weight of 29 kDa of major acid phosphatase isoenzyme secreted by maize endosperm culture has also been reported (Miernyk, 1992). Similarly one isoenzyme of 18 kDa was also purified from *Hypericumperforatum* (Demir*et al*., 2004). Gonnety*et al*., (2006) also isolated three isoenzymes with molecular weights of 22, 24 and 25 kDa from peanut seedling. Generally the molecular weights found for acid phosphatases from other sources were between 13 kDa and 100 kDa (Felenbok, 1970) and most of the enzymes were found to be glycoproteins and these had similar molecular weights ranging from 50 kDa to 60 kDa (Olczak*et al*., 1997., Gellatly*et al* ., 1994., Pasqualini*et al*., 1997).

The optimal pH for this enzyme was 5.5 and pH stability was 4-7. It had optimum temperature of 60^{o}C and temperature stability was 0-50^{o}C. Metal ions such as Cu^{+2}, Hg^{+2}, Zn^{+2} and Al^{+3} showed strong inhibition while other monovalent and divalent ions like Na^{+}, K^{+}, Pb^{+2}, Ba^{+2}, Cd^{+2}, Mn^{+2},Ca^{+2}, Mg^{+2} and Co^{+2} were found no effect on the enzyme activity .Modifiers, Reducing agents, Detergents and Anions were also found to have no effect on the enzyme activity.

The kinetic study showed a linear Lineweaver-Burk plot with a K_m of 0.3 mM for pNPP. This value was very similar to that of acid phosphatases of yellow lipin seeds (Olczak*et al*., 1997), group III isoenzyme of rice plant (Tso and Chen, 1997) and soyabean seeds (Kaneko *et al*., 1990). It was also consistent with the K_m value of the barley root acid phosphatases which was reported to be pH dependent and at pH 5.5, it was about 0.3 mM (Arnold *et al* .,1987).

Acid phosphatase from leaves of *V. radiata* like other plant acid phosphatases was competitively inhibited by inorganic phosphate (Olczak*et al*., 1997, Kaneko *et al*., 1990, Ullah and Gibsson, 1988, Cirkovic *et al*., 2002). It indicates that feedback inhibition of acid phosphatases by inorganic phosphate may represent a general form of cellular regulation of these enzymes (Duff *et al*., 1994, Olczak*et al*.,1997). Acid phosphatase enzyme was inhibited non-competitively by fluoride while molybdate showed mixed type inhibition. Similar results have been reported by Keneko *et al.,* 1990. Vanadate, an inhibitor of acid phosphatases (Tabaldi *et al*., 2008) also inhibited this enzyme competitively. The molybdate was four times more powerful inhibitor than vanadate. These results were consistent with that of Kaida *et al* 2008. Similarly, zinc was found non-competitive inhibitor of this enzyme. Non-competitive inhibition was also observed in acid phosphatses purified from seedlings of soybeans (Kaneko *et al*., 1990).While mercury was found an un-competitive inhibitor of acid phophatases. But the enzyme was found insensitive to tartrate inhibition. This tartrate resistance was also found in many other plant acid phosphatases (Olczak *et al*., 1997, Cirkovic*et al*., 2002). This enzyme may also be recognized as tartrate resistant acid phosphatase.

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